SOME QUESTIONS RELATING TO THE VIRULENCE OF MICRO-ORGANISMS WITH PARTICULAR REFERENCE TO THEIR IMMUNIZING POWERS.

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PLATES XII–XX.

The experimental work which forms the basis of this article was for the greater part performed during the spring of 1903, in the Institut für Infektionskrankheiten, Berlin, Professor R. Koch, Director, and in the department of Professor A. Wassermann. I wish here publicly to express my very grateful thanks to Professor Wassermann, under whose direction these studies were first undertaken, for many suggestions and courtesies.

During an experimental study of protective inoculation against Asiatic cholera, it became desirable to conduct experiments with two strains of cholera spirilla of different degrees of virulence. Throughout the course of these studies, which extended over a period of a number of months, there often arose in my mind the question of the essential differences existing between these two stems, particularly in relation to the subject of their virulence and to the immunity to which they gave rise in inoculated animals. The investigation of these questions forms the basis of this paper.

For the description of the two strains, their source, identification, etc., the details of the technique employed in the experiments, and other particulars, the reader is referred to a previous article (1).

It will be sufficient to state here that some time was spent in accurately standardizing these cultures, and the minimal lethal dose for guinea-pigs weighing 250 grams was carefully

determined.[1] After numerous passages of "Virulent" through animals, the lethal dose of 0.1 of a standard (2 mg.) oese [2] of a twenty hours' agar culture was reached. Such a dose of "Virulent," when suspended in 1 c. c. of a 0.85% sodium-chloride solution and injected intraperitoneally into a guinea-pig of 250 grams' weight, regularly caused death within twenty-four hours; with "Avirulent," on the other hand, one and a half standard oesen of a twenty hours' agar culture, when injected intraperitoneally, was required to produce death within the same time in such an animal. Therefore the former stem may be said to possess fifteen times the virulence of the latter.

Throughout the course of the work this relation of virulence was carefully maintained. As the virulence of cholera spirilla which have been grown upon artificial media quickly changes, it was necessary in the case of the virulent organism to make daily animal inoculations and always to use the same generation of the strain. With the avirulent culture considerable care was also required to keep it at the minimal lethal dose of one and a half oesen.

TECHNIQUE.

The technique of the agglutinative and bactericidal reactions employed throughout the work was as follows:

The reactions for agglutination were performed in the test-tube. One oese of the living organisms was thoroughly suspended in 1 c. c. of a 0.85 % solution of sodium chloride. The amount of serum, to be tested, suspended in 1 c. c. of a similar saline solution was then added, the tube thoroughly shaken, and the mixture placed for two hours at 37° C. By a complete agglutination it is understood that the liquid overlying the precipitated bacteria appears entirely clear. By a weak reaction we understand one in which there is a distinct agglutination with precipitation, visible to the naked eye, of numbers of the organisms, but in which the supernatant fluid remains more or less cloudy.

The bactericidal reactions were performed in the abdominal cavity of guinea-pigs according to the well-known method of R. Pfeiffer, a hypodermic syringe with a blunt-pointed needle being employed for the injections, and care being taken to avoid any injury to the intestine during the inoculation. The dilu-

[1] For the sake of brevity these strains will, in this article, be designated as "Virulent" and "Avirulent."

[2] This standard oese was employed throughout the work.

tions of the serum were made in normal saline solution. One c. c. of the diluted serum was added to 1 c. c. of bouillon containing two oesen of "Virulent" in suspension, after which 1 c. c. of the resulting mixture was injected into the peritoneal cavity of a guinea-pig of 250 grams' weight (or a little less), the animal thus receiving ten times the fatal dose of living organisms. A fresh guinea-pig was used for each reaction. The experiment was controlled by microscopic examination of a drop of serum from the abdominal cavity, made immediately and again twenty minutes after the inoculation, and obtained by means of a capillary tube, and by the inoculation of control animals with ten times the fatal dose of "Virulent" without serum. The result to the animal after twenty-four hours, whether living or dead, was regarded as the final test, though the condition of the organisms in the abdominal cavity after twenty minutes was always carefully noted.

With these explanations we may now turn our attention to the study, between the two stems, of certain of the essential differences in relation to virulence.

By the virulence of a micro-organism we have come to understand its pathogenic capabilities—that is, the extent to which it may harm a susceptible host. The virulence of a bacterium, therefore, represents the sum of its specific injurious influences upon such a being. Some authors differentiate between virulence and toxicity (2), including in virulence only the infectious capability of the organism—that is, the power to grow and to multiply in the animal body,—and in toxicity the ability to produce a specific poison and the amount of such poison. However sharply such a distinction may be observed in diphtheria and tetanus, in which diseases the infectious process (i.e., the propagation of the living bacteria) and the intoxication process (that one due to the action of the soluble non-viable substances set free from the organisms) are quite distinct,[3] and while it is well known that certain strains of diphtheria bacilli may at the same time possess strong infective and but little toxic power (and vice versa), in Asiatic cholera the two processes of infection and intoxication are apparently so closely intermingled that, although theoretically a sharp distinction is possible, practically, in the study of the infection of the animal organism, it is only with difficulty that the two are

[3] With the exception, perhaps, in diphtheria, of the intracellular toxine of Welch and Flexner, concerned in the production of the false membrane.

actually differentiated. Hence, in considering the virulence of a strain of living cholera spirilla, both its infectious and its toxic power may, to a certain extent, be regarded conjointly.

Until recently our ideas as to what specific properties the virulence of an organism depends upon have been very vague and indefinite.

Kruse (3), in his earlier publications regarding the theory of infection and immunity, maintained that a certain analogy existed between the virulence of micro-organisms and their ability to produce enzymes; he conceived the hypothesis that pathogenic bacteria possess a certain dissolving power ("lytische Kräfte"), through which they are able to bind and to paralyze the opposing bodies (alexines) of the living organism. A loss of virulence was therefore supposed to go hand in hand with a loss of these dissolving substances ("Angriffsstoffe").

Smirnow (4) pointed out that cultures which show a loss of potency in their vital manifestations (especially in their virulence) also present simultaneously a diminished energy of growth. Thus, he maintained that the diameters of colonies of virulent anthrax bacilli are from two to four times as great as those of avirulent strains of the same organism. However, he shows that the diminished virulence of a bacterium depends not alone upon the loss of some one specific property but upon a real degeneration of the organism, which manifests itself by a diminished energy of growth and a greater susceptibility to damaging influences.

Behring (5), in working with different strains of anthrax bacilli, in general confirmed Smirnow's observations. He further pointed out that in cultures of this organism the production of acid as well as the power to reduce litmus increases with the virulence of the strain.

Gotschlich and Weigang (6) maintain that the virulence of a cholera culture depends exclusively upon the number of living individual spirilla which it contains. According to their view, the virulence of an organism would depend upon its power to multiply within a given time—that is, the more virulent the organism the shorter the interval between successive generations.

Beyer (7) found that, if a small piece of silver foil was placed upon the surface of an inoculated agar plate, the more virulent the organism the narrower was the clear zone between the growth and the silver foil. In case the organism was very virulent, the growth was said to extend so as to touch the margin of the silver.

Marx and Woithe (8) stated that the virulence of a micro-organism in a human or in an animal infection can be judged by the number of bacteria which contain Babes-Ernst's bodies. The more numerous such organisms are in a disease or in a culture the greater is its virulence. However, Ascoli (9), Krompecher (10), and Gauss (11) were not able to confirm this observation. Thus, Gauss, using a culture of bacillus pyocyaneus, which had been passed many times, through animals, obtained a virulence forty times as great as that of the original strain. Yet he was unable to find Babes-Ernst's bodies in a single organism of this culture, which possessed the highest obtainable virulence.

Pfeiffer (12) in 1897 found that in immunization against plague the degree of immunity produced depended not only upon the dose of the killed pest culture but also upon the degree of virulence of the killed organism. He showed that an ape, which had been given a single injection of a virulent agar culture, carefully sterilized by heating, was later protected against one oese of the virulent bacterium. If, however, an avirulent strain of the pest culture was employed for the virus, the animal was not protected against the same amount of the virulent germ. Therefore Pfeiffer concluded that the immunizing effect of pest bacilli is, up to a certain degree, proportional to the virulence of the culture employed. Pfeiffer and Kolle (13) in 1895 also demonstrated that a virulent typhoid bacillus required many more times the amount of immune serum to bring about its bacteriolysis in the abdominal cavity of a guinea-pig than did the less virulent strain. In 1896 these authors (14) pointed out that an immune serum agglutinated a less virulent cholera organism in much higher dilutions than a virulent one. Pfeiffer (15) continued his researches in this direction, and further

investigated with Friedberger (12) the question of the virulence of the cholera vibrio. They concluded that in the case of the killed cultures of this organism the immunizing effects were also proportional to the virulence of the inoculated strain. From these facts they drew the conclusion that the virulent and the avirulent organisms differ in the number or degree of affinity of their haptophore groups and demonstrated this conclusion by experiments in which it was shown that in a goat's cholera immune serum a virulent organism bound many more times the number of amboceptors than certain avirulent ones.

These last experiments of R. Pfeiffer and Friedberger seemed of such great importance in connection with Ehrlich's hypothesis that it was decided to repeat them, and, in addition, to perform them in as accurate a comparative way (with relation to the virulence of the strain) as practicable. This seemed desirable because in Pfeiffer and Friedberger's work, as far as can be ascertained from their article, no attempt had been made previously to determine the exact relationship of virulence of the different stems to one another, it being apparently the authors' intention to show in these experiments that the virulent organism always bound more amboceptors than the less virulent ones; at any rate, the exact relationship between the virulence and the power of binding amboceptors was not emphasized.

Moreover, Pfeiffer and Friedberger's absorption experiments above referred to were performed with the serum of immune goats, while the bactericidal reactions were performed in the abdominal cavity of guinea-pigs. Since at the time these experiments were performed it was not established whether the receptor structure of the cholera vibrio for both of these animals was identical,[4] and whether the difference in virulence between the two strains was relatively the same for each animal, and since it was even disputed whether their intermediary bodies were identical, it was thought desirable to perform the experi-

[4] Since these experiments were performed, Pfeiffer and Friedberger (16) have shown that goats' and rabbits' cholera amboceptors unite with the same receptors of the cholera spirillum.

ments also with guinea-pig serum. This work was undertaken in the manner to be described.

ABSORPTION EXPERIMENTS WITH THE LIVING ORGANISMS AND GUINEA-PIG IMMUNE SERUM.

A large guinea-pig was inoculated subcutaneously with 2 c. c. of an aqueous solution containing the free receptors of the virulent cholera spirillum obtained by the autolytic digestion of this organism,[5] and eight days later was re-inoculated intraperitoneally with two oesen of the living virulent strain. After another eight days it was killed by bleeding and the bactericidal value of its serum carefully determined. This was found to be about 0.11 mg. Separate portions of the serum were then diluted with normal saline solution in the proportions of 1: 20 and 1: 100. Four centrifuge tubes (designated for convenience as A, B, C, and D) were then taken. Two of them, A and B, were filled with 5 c. c. of the serum diluted in the proportion of 1:20; the other two, C and D, with 5 c. c. of the serum diluted in the proportion of 1: 100. Five oesen of the living virulent organism were then suspended in the serum of each of tubes A and C, and five oesen of the living avirulent organism in each of the two remaining ones, B and D. After a thorough mixing of the contents, the tubes were placed for two hours in the ice-box. This time was allowed for the binding reaction between receptors and amboceptors to become complete; and the object of keeping the tubes at a low temperature was to prevent any further multiplication of the spirilla. Upon removal of the tubes from the ice-box the agglutination of the organisms was apparently complete in all of them. After thorough centrifuging, the clear fluid above was pipetted off and in each case carefully examined for its bactericidal value in the usual manner. Table IX* shows these results *calculated for 1 c. c. of the undiluted serum.*

From a study of the serum diluted to 1:20, we see that the portion treated with the virulent strain afterwards showed a

[5] This solution constitutes our cholera prophylactic. For a detailed description of its preparation, etc., the article on "Protective Inoculation against Asiatic Cholera" (1) above referred to should be consulted.

* The "Tables" are given as plates at the end of this article.

bactericidal value of 1:500 or about one-seventeenth of that treated with the avirulent one (1:8500); and upon subtraction of these values from that of the original serum (1:9000) we see that the virulent organism has bound about $\frac{85}{90}$ and the avirulent one about $\frac{5}{90}$ of the amboceptors present. In the case of the serum diluted to 1:100, the bactericidal value of the portion treated with the virulent strain (1:600) was about one-fourteenth as great as that treated with the avirulent one (1:8500), the *ratio of absorption* being as 84:90 to 5:90. Hence, evidently in the cholera immune serum the virulent organism had bound from sixteen to seventeen times as many bacteriolytic amboceptors as the avirulent one; or the haptophore groups of the former spirillum had shown from sixteen to seventeen times as great a power of absorption as those of the latter.

The results of these experiments, showing the values of the sera expressed in units of immunity for 1 c. c. of the diluted serum and the number of units absorbed per c. c., may be seen in the accompanying tables.

Dilution of the serum to which the bacteria were added.	Original value of 1 c. c. of the diluted serum in units of immunity.[6]	Value of 1 c. c. of the diluted serum (in immunity units) after the absorption of 1 oese per c. c. of	
		Virulent.	Avirulent.
1:20	450	25	425
1:100	90	6	85

Dilution of the serum to which the bacteria were added	Original value of 1 c. c. of the diluted serum in units of immunity.	Number of units absorbed per cubic centimeter by 1 oese of	
		Virulent.	Avirulent.
1:20	450	425	25
1:100	90	84	5

[6] By one unit of immunity we understand the amount of serum which will protect a guinea-pig of 250 grams' weight against 10 times the fatal intraperitoneal dose of living cholera spirilla. Throughout the course of the work only the "virulent" organism was employed in testing the bactericidal power of the sera.

ABSORPTION EXPERIMENTS WITH THE LIVING ORGANISMS AND RABBIT IMMUNE SERUM.

Experiments were then performed in a parallel manner with rabbit immune serum, which was obtained in the following manner: A rabbit was inoculated with 6 c. c. (1 c. c. contains the number of receptors obtained from eight oesen) of the heated virulent cholera prophylactic. Eight days afterwards it was killed by bleeding, and its blood serum upon examination was found to have a bactericidal value of .04 mg. Five oesen of the living virulent strain were now carefully suspended in 5 c. c. of this serum in dilutions of 1:20, 1:100, and 1:500. Five oesen of the avirulent organism were also added to each 5 c. c. of the serum in the same dilutions. The mixtures were placed in the ice-box for two hours. Upon removal it was found that in the tube which contained the serum in the dilution of 1:500 and the virulent organism, agglutination was not entirely complete, and that, although the overlying liquid was nearly clear, it was not wholly so, and evidently still contained some organisms. In the other tubes complete agglutination of the bacteria had apparently occurred. The mixtures were then carefully centrifuged and the overlying liquid was pipetted off, and in each instance examined for its bactericidal value by the usual method. The results are recorded in Table X (as calculated for 1 c. c. of the undiluted serum), and are in general similar to those obtained in the case of the guinea-pig serum. The values of the sera expressed in units of immunity for 1 c. c. of the diluted mixtures are given in the tables which follow.

The second table shows the number of units absorbed per c. c. by one oese of the organism.

Dilution of the serum to which the bacteria were added.	Original value of 1 c. c. of the diluted serum in units of immunity.	Value of 1 c. c. of the diluted serum (in immunity units) after the absorption of 1 oese per c. c. of	
		Virulent.	Avirulent.
1:20	1200	50	1120
1:100	240	12	225
1:500	48	10	45

Dilution of the serum to which the bacteria were added.	Original value of 1 c. c. of the diluted serum in units of immunity.	Number of units absorbed per c. c. by one oese of	
		Virulent.	Avirulent.
1:20	1200	1150	80
1:100	240	228	15
1:500	48	38	3

From these tables (and Table X) we see that in the dilution of 1:20 the virulent organism had bound about fourteen times as many amboceptors as the avirulent one ($\frac{230}{240}$ to $\frac{16}{240}$), and in the dilution of 1:100 about fifteen times as many ($\frac{228}{240}$ to $\frac{15}{240}$). In the dilution of 1:500, the serum, after treatment with the virulent organism, showed a value of 1:5000 (0.2 mg.); while that previously treated with the avirulent one showed a value of 1:22,500 (nearly .04 mg.). Therefore the value of the latter serum was less than five times as great as that of the former, the ratio of absorption being $\frac{15}{240}$ to $\frac{190}{240}$, or about 12 to 1. However, the experiments with the serum dilution of 1:500 might be misleading without the following explanation.

As already pointed out, the agglutination of the virulent organism in the dilution of the serum of 1:500 was not complete, even after the mixture had stood for two hours at the temperature of the ice-box, and after prolonged centrifuging the overlying fluid was still not entirely clear. Evidently there still remained in this supernatant fluid above the precipitate a few vibrios, in which a sufficient number of the haptophore or agglutinophore groups of the agglutinable substances necessary to bring about the phenomena of agglutination were not yet bound by their respective groups of the agglutinin. When this serum was injected into guinea-pigs, it clearly carried with it not only the *free* bacteriolytic amboceptors of the serum but also those bound to the bacteria and remaining in the slightly cloudy fluid. These amboceptors, meeting with a suitable complement in the abdominal cavity of the guinea-pig (and combined with an additional number of amboceptors if necessary), obviously destroyed the bacteria to which they were already

united by their haptophore groups before inoculation; and in this manner (the amboceptors), being set free, were capable of again unfolding their bacteriolytic action against one oese of the fresh living organism introduced for the regular bacteriolytic test. Hence, owing to the combined action within the abdominal cavity of the guinea-pig of the *free* amboceptors in the serum introduced and of those carried in with it *bound to the bacteria*, the value of the serum appeared higher than it would have done, if the agglutination of the organisms by it had been complete and they had been in this manner originally separated from the mixture.

In support of this argument it may be seen from the experiments shown in Table X that, while in the dilution of 1:500 of the serum, after treatment with the virulent strain (where agglutination was not complete), we find a bactericidal value of 0.2 mg. (1:5000), in the dilution of 1:100, after treatment with the virulent organism (where agglutination was originally complete), we find a value of only 0.8 mg. (1:1200).

The actual bacteridical value of this serum (dilution of 1:500), in which the agglutination of the virulent organism was not complete, could not be more accurately determined after the removal of the incompletely agglutinated bacteria by filtration, since in this process, while the combined amboceptors were separated, a portion of those unbound also remained behind on the filter.

In this same dilution of the serum (1:500) with the avirulent organism, the agglutination of the spirilla being complete on account of the fewer agglutinable receptors necessary to be occupied in order to bring about this phenomenon, no bacteria-binding amboceptors were carried into the abdominal cavity of the guinea-pig and the value of the serum was practically the same (viz., 1:22,500) as in the the lower dilutions of 1:20 and 1:100.

That the explanation thus given is applicable to these results is confirmed by the very important research of R. Pfeiffer and Friedberger (16), published, however, several months after the experiments mentioned above were completed. These authors

show conclusively that the cholera amboceptors bound to cholera spirilla were not destroyed, either in the event of the death of the organisms (bacteriolysis) or in that of their subsequent life, but that they were in both instances eventually set free from the bacteria and again became capable of exercising their bacteriolytic power. A catalytic action is therefore suggested.

On comparing the results of Tables IX and X, emphasis is again laid on the fact that in the cholera immune serum of both rabbits and guinea-pigs the virulent organism bound from fourteen to seventeen times as many bacteriolytic amboceptors as the avirulent one. This difference in the power of binding practically corresponds to the difference in virulence between our two strains, "Virulent" and "Avirulent." Hence, these experiments bear out the hypothesis that the virulence of a living cholera organism is proportional to the number or degree of affinity of its bacteriolytic haptophore groups.

Another point which is demonstrated by this investigation is that the organisms of each strain bind proportionately the same number of amboceptors in the rabbit cholera immune serum as in that of the guinea-pig. This suggests that the cholera amboceptors of rabbits and guinea-pigs unite to the same receptors of the cholera vibrio, i.e., that the receptors of this organism are identical for both animals. Pfeiffer and Friedberger (16), since these experiments were performed, have shown by an entirely different method of experimentation that the receptors of the cholera spirillum are identical also for goats and rabbits.

The avirulent organism was next passed successively through the abdominal cavities of about twelve guinea-pigs and then examined in regard to its virulence. This was found to have considerably increased, since now three-fourths of an oese of the organism produced death in a guinea-pig of 250 grams' weight within twenty-four hours. Unfortunately, at this time there was no longer on hand any serum from either of the animals with which the series of experiments given in Tables IX and X were performed, so that a comparative study could not be made. However, with another rabbit cholera immune serum, to which

were added corresponding amounts, first, of the original avirulent strain (of one and a half oesen virulence), and secondly, of the avirulent strain after about twelve successive passages through guinea-pigs (of three-fourths of an oese virulence), it was found that the latter strain was able to bind in the immune serum nearly twice as many amboceptors as the former. In other words, with an increase in the virulence of the organism, an increase in the number or binding power of its haptophore groups had occurred.

It would be interesting to follow this relationship quantitatively to its logical conclusion, and to plot the result as a curve; in this way a mathematical basis of the relation between receptors and amboceptors might be obtained, and light thrown on the nature of this relation. This work will be shortly undertaken in this laboratory.

COMPARISON OF THE AGGLUTINATION OF THE VIRULENT AND AVIRULENT STRAIN.

In the work on protective inoculation against cholera, already referred to, it became evident that the avirulent organism was agglutinated by higher dilution of the same sera than the virulent one. This may be seen in Tables I to VIII of this article. However, it is true that sometimes the difference in agglutination varied, owing to the fact that the serum used was not always of the same age; and in case varying amounts of agglutinoid were present, the usual ratio of agglutination between the two stems was lost, because smaller amounts of agglutinoid prevented agglutination from appearing in suspensions of the avirulent than in suspensions of the virulent organism. Theoretically these results could be explained on the assumption that there existed fewer agglutinable haptophore groups in the avirulent than in the virulent strain, and hence a smaller number of uniceptors was necessary to bring about a reaction in the case of the avirulent germ than in that of the virulent one, so that with the former organism agglutination took place in higher dilutions. Likewise, when sufficient agglutinoid was present, smaller numbers

of such modified uniceptors would suffice to bind the receptors of the avirulent strain than would be required by the virulent one. Hence the phenomenon of agglutination would fail with the avirulent organism in lower dilutions than it would in the case of the virulent one. However, even in a fresh cholera immune serum no such difference as 15:1 could ever be demonstrated in the agglutinable power of the two stems—that is, the avirulent strain was not agglutinated in dilutions fifteen times higher than those necessary in the case of the virulent race. Neither could it be shown with the same sera (used in the experiments of Table X) that the virulent strain bound fifteen times more agglutinin than the avirulent, though it was true that the amount of agglutinin which the former organism appropriated was about four or five times as great. We may then argue that, while the virulent organism contains more agglutinable substance than the avirulent, i.e., that its agglutinable haptophore groups are more numerous, its virulence (from an infectious point of view) is not in direct proportion to the amount of such substance, and just as the amount of agglutinin in a serum has not been found to be directly proportional to the degree of (at least the bactericidal) immunity of the host, so it may now be stated that neither is the amount of agglutinable substance directly proportional to the (infectious) virulence of the organism. Obviously we must not lose sight of the fact that, just as in the immunity of the host, two, and perhaps three, factors may enter into consideration, namely, the anti-toxine, the bacteriolysin, and the agglutinin, so, in the question of the virulence of a cholera organism, there may also be three substances to be considered, namely, the toxin, the bacterial cell, and the agglutinable substance.

From our study as to what properties the virulence of our two cholera stems depends upon, and from our recorded animal experiments, it seems that it is the power of the cell to bind bacteriolytic amboceptors and to resist destruction (bacteriolysis) as well, which is the most important element connected with the death or recovery of the animal (our indicator of the virulence); and that it is the ratio of this power (both to bind

and at the same time to prevent bacteriolysis) rather than that of any other which we express, when we state that the virulence of the organisms is as 15:1. Therefore, even should the agglutination proceed in a manner parallel to that of the bacteriolysis, it is questionable whether we should expect a priori that our virulent organism would bind in the immune serum fifteen times more agglutinin than the avirulent; since it has not been demonstrated (even granting for the moment that the amount of agglutinable substance which the organism possesses is a possible preliminary factor entering into the question of the virulence) that the ratio of the amounts of the agglutinable substance contained in the two stems is as 15:1. Indeed, in so far as the amount of this substance could be demonstrated from the action of the strains upon an immune serum, the avirulent organism was never agglutinated in dilutions higher than about five times those which agglutinated the virulent strain. However, Eisenberg and Volk (17) thought that it was doubtful whether the agglutinable substances of an organism could be fully saturated, since they were able to find no limit to this power. Moreover, it is well known that the more concentrated the serum, in agglutinin, the greater is the quantity of agglutinin bound by the same amount of agglutinable substance. The question of the velocity of the reaction should, therefore, be carefully considered in relation to this phenomenon.

In connection with this subject, the very recent work of Arrhenius (18) is also of interest. This author found that for constant quantities of bacteria, the amount of free agglutinin being B and the amount of bound agglutinin C, the following relation exists: $C = \text{Konst. } B^{\frac{1}{2}}$. However, if the quantity of bacteria (A) varied, the following equation was found to exist, viz., $\frac{C}{A} = KB^{\frac{1}{2}}$. That is, the absolute quantity of bound agglutinin did not enter into the question, but only its concentration in its solvent—the bacteria. He pointed out that, with a knowledge of the conditions of equilibrium, the assumption that the agglutinin exists as a number of substances with binding properties of different degrees of affinity is superfluous, even if

the possibility that the agglutinin is a mixture of several active bodies cannot be denied.

Arrhenius apparently worked with a single strain of an organism of a certain virulence. It would be very interesting to perform these same experiments with strains of different virulence and with the free agglutinable receptors of such organisms. We might expect, a priori, that the same law would apply in such experiments, since by the "quantity" of the bacteria we probably really understand the number of agglutinable haptophore groups, which, in the case of the virulent organism, would be greater than in that of the less virulent strain. Hence $\frac{c}{\lambda}$ would vary with the virulence of the organism.

However, we must return to the question of the virulence of the two strains of cholera spirilla and defer for the present any further discussion of this matter.

ABSORPTION EXPERIMENTS WITH THE KILLED ORGANISMS AND RABBIT IMMUNE SERUM.

With the hope of throwing more light on this relation, a study of the effect of the absorption of the amboceptors from the same immune serum by means of the killed organisms was undertaken. The minimal lethal dose, for guinea-pigs, of the spirilla killed with chloroform was first carefully determined for each strain. It was found that about five or six oesen of the killed virulent organism, when introduced into the abdominal cavity, produced death within twenty-four hours in guinea-pigs of 250 grams' weight, while about eight or nine oesen of the killed avirulent strain was necessary to cause the same result. Therefore, with the killed organisms a ratio of virulence of nearly 2:1 existed.[7] These results speak partly in favor of the hypothesis of Gotsch-

[7] It may be justly argued that this is merely a toxic ratio and not one of virulence. However, if we assume that the toxin is intracellular, the binding power of each strain of the killed organisms for bacteriolytic amboceptors, as will be pointed out further on, is apparently of some importance in the liberation of this toxin, and since the bacteriolytic receptors are partially concerned in this liberation, it has been thought advisable to employ the term virulence (in this connection) as representing the injurious influence of the organism upon a susceptible being (the guinea-pig). It is admitted that ordinarily the terms virulence and toxicity may with advantage be distinguished from one another.

lich and Weigang (6), though from them it is evident that if the virulence of a living organism depended only upon its power to multiply more or less rapidly within a given time, the virulence of our two strains of cholera spirilla (provided that exactly the same amounts of each were used) would probably be equal after the death of the organisms. However, this, as we have just seen, is not the case, a difference in virulence of nearly 2:1 existing between the two killed races. On the other hand, in the living state a difference of virulence between the organisms of 15:1 existed. Therefore, these experiments would support the idea that the energy of growth of an organism is a factor of importance, though not the only one, in relation to its toxic virulence.

For the absorption experiments with the killed organisms the same rabbit cholera immune serum was used as was employed for those recorded in Table X with the living spirilla.

Five oesen of each strain, the virulent and the avirulent, were suspended in 5 c. c. of bouillon in separate test-tubes, to each of which were then added fifty drops of chloroform. After the organisms had been killed, the chloroform was evaporated, the sterility of the mixtures was demonstrated, and then there were added to each of the two tubes 5 c. c. of the rabbit serum in dilutions of 1:20. Hence the dilutions of the serum in each of the two tubes containing five oesen of the organisms equalled 1:40. The mixtures were allowed to stand for two hours, and after complete agglutination had occurred in both of them, the clear fluid above was pipetted off and examined in each instance for its bactericidal value. The results of these experiments calculated for 1 c. c. of the undiluted serum may be seen in Table XI. We first notice that the receptors of the organism have been seriously injured or diminished through the killing of the spirilla by chloroform; for, whereas the living virulent organism bound about $\frac{23}{24}$ of the bactericidal amboceptors in the same rabbit serum, the killed virulent organism was able to bind only about $\frac{1}{24}$ of them.[8] This loss or injury of receptors apparently

[8] In making this comparison, we must assume that the living bacteria in the experiments of Table X had not multiplied during the time that they were in contact with the serum (2 hours) at the temperature of the ice-box.

progresses to a certain extent with the loss of virulence, the minimal lethal dose of the virulent strain being for the killed organism about five or six oesen and for the living one-tenth of an oese. However, while the ratio of virulence between the killed and the living germ may be expressed as about 1:55, the difference in their power to absorb the bacteriolytic amboceptors is not at all in this proportion. Hence, it would appear that the relation between the virulence of the killed organism and that of the living one, both belonging to the same strain, is not always, at least, dependent upon the number or binding power of the bacteriolytic haptophore groups possessed by each, or at any rate not dependent alone upon this condition. In other words, it would seem that in the death of the bacteria by the process described a certain change has taken place in the organism, so that while the receptors may be able to bind in vitro a considerable number of amboceptors, the poisoning action of the bacteria in the animal body is not unfolded to the extent which might be expected. Therefore the idea is suggested that in the killing of the organism with chloroform the intracellular toxin has also suffered a change—that is, it now has been placed in such a condition as not to be so easily set free from the bacteria (possibly through a retarding of the action of the ferments of the organism), or it has actually been altered chemically. In other words, in the case of the killed bacterium entirely another factor besides its power of binding amboceptors and its resistance to destruction (bacteriolysis) would seem to enter principally into the question of virulence (or the fate of the inoculated animal), namely, the condition and the amount of the intracellular toxin set free.

We have seen that with the killed virulent organism amounts as large as five or six oesen are necessary to bring about the death of a guinea-pig. On the other hand, it has been demonstrated by experiments in which the killed organisms were added to the immune serum in vitro, that the killed bacteria still contained a considerable number of the haptophore groups, as the proportion of the amboceptors removed from the serum demonstrates. Indeed, they were capable of binding many more

amboceptors than would be necessary in order to bring about a complete dissolution of the bacteria in the animal body. Hence, upon their introduction into the abdominal cavity of an animal, probably the factor of the greatest importance in relation to its death or recovery would be the condition and the amount of the intracellular toxin which the bacteria contained. The virulence in this instance would chiefly depend upon this value.

Possibly the slight and partial injury by chloroform of the agglutinophore group of the agglutinable substance in the dead bacteria may also be a preliminary factor which exercises a retarding effect upon the virulence, by placing the organism in a less satisfactory condition for the liberation of the toxin.

After a comparison of the values of the sera following treatment first with the living and then with the killed avirulent organism (see Tables X and XI), we are inclined to the same explanation, though some of the results cannot be satisfactorily interpreted. Thus, it must be stated here that even when a smaller number of receptors was placed in this immune serum, it was found that generally a loss of amboceptors of from about .001 mg. to .003 mg. per cubic centimetre occurred in the undiluted serum; that is, when the number of receptors existing in the avirulent organism became very small, they seemed endowed, upon being placed in a concentrated serum, with a slightly greater binding power. Thus, in this series of experiments, with the avirulent organism, the killed germ absorbed apparently the same number of amboceptors as the living one, though in each case this was actually very small. However, the ratio of virulence of the living germ to the killed one was about 5.5:1 (1.5 to 8 or 9 oesen).

On comparing the value of the serum added to the killed virulent organism with that of the one added to the killed avirulent organism, we see that the former has about one-fourth the strength of the latter; or, that the virulent organism has bound $\frac{19}{24}$ and the avirulent one $\frac{5}{24}$ of the bacteriolytic amboceptors of the serum. Beginning, then, with a ratio of virulence of about 2:1, we obtained an absorption ratio of (bacteriolytic) amboceptors of about 9:1; that is, the virulent

organism bound nine times as many amboceptors as the avirulent one. However, these results are not confusing, since it has already been pointed out that the ratio of 2 : 1 is mainly a ratio of the toxic haptophore groups, while that of 9 : 1 is a ratio of the bacterial haptophore groups of the two strains. But, as has been pointed out above, even when such an absorption of the amboceptors by these respective organisms occurs in the animal body, an absorption which is evidently far greater than that necessary for the complete dissolution of the bacteria and the liberation of the toxin, the death of guinea-pigs will not result with smaller doses than five or six oesen of the killed virulent organism, and eight or nine oesen of the killed avirulent one; for the reason that in smaller amounts of the bacteria (killed after this manner) there is not present a sufficient amount of the unchanged toxin to accomplish this end. This once more forces us to the conclusion that the difference in virulence between the organisms killed with chloroform *in this manner* is not alone dependent upon the number or the binding power of the bacteriolytic haptophore groups, but also upon the number and binding power of the toxic haptophore groups, i. e., the amount and the condition of the intracellular toxin present in the organism at the time of its inoculation.

On the other hand, the virulence of the killed organism may depend to a certain extent upon the number or the avidity of the bacteriolytic haptophore groups, since the greater the number present or the greater their binding power, the larger the quantity of amboceptors excited and then bound (within a given time), and hence the quicker the complete dissolution of the bacteria and the greater the amount of toxin liberated within a given moment, and therefore the greater the injury to the animal. We have already seen that in the killed virulent strain the bacteriolytic haptophore groups are actually much more numerous or endowed with much greater binding power than in the killed avirulent one, but in this connection it must again be noted that it is not a question of the bacterium being killed by the amboceptors (death has already taken place), but it is their dissolution which we suppose results with the viru-

lent strain within a shorter period of time. Were we considering the living bacteria, the hypothesis would necessarily be somewhat different.

The living virulent organism evidently has greater powers of resistance than the avirulent, and more amboceptors are required for its destruction, but through the possession of an increased binding power in its haptophore groups, and hence its greater avidity for amboceptors, it is more capable both of appropriating and of giving rise to the production of these groups in the animal body than is the avirulent germ. Therefore, upon the entrance of the virulent bacteria into a susceptible individual, a number of the organisms become more quickly destroyed by means of this power to absorb whatever amboceptors are present or are produced at the time of their introduction. The intracellular toxin of these organisms is thus liberated. However, since a sufficient number of amboceptors to satisfy the avidity of all the organisms is not at once produced, the remaining living bacteria during this latent period multiply rapidly through the increased energy of growth which the virulent organism possesses. As soon as the animal body has responded to an additional production of amboceptors or a sufficient number are set free from the bacteria which have already been killed, an additional number of the virulent organisms are bound and destroyed, and a fresh intoxication of the host results. Hence, the virulence of the living cholera spirillum depends, probably, both upon its power of resistance to the amboceptors and its power to excite and to absorb these substances, as well as upon the amount of intracellular toxin (the number of toxic haptophore groups) it possesses and its energy of growth.

In this connection it is well to call attention to the work of Von Dungern (19), who concluded, from a series of inoculations in animals, that the virulence of two strains of cholera spirilla was independent of their toxic properties. However, it hardly seems that one would be justified in drawing such a conclusion from Von Dungern's experiments. It would appear, at least from his results with the intraperitoneal inoculation of guinea-pigs with the killed organisms, that the virulent organism was

more toxic than the less virulent one, though it is true that there was certainly a great difference in the ratio of virulence when compared with the ratio of toxicity, the latter being nearly identical. These results, however, practically coincide with our own, namely, that with a difference of virulence of 15:1 with the living organisms, we obtained a toxic ratio with the dead strains of less than 2:1.

It now seemed desirable to study the effect upon this immune serum of the free bacterial receptors of the cholera spirillum in solution, obtained by autolytic digestion and prepared both from the virulent and from the avirulent strain.

ABSORPTION EXPERIMENTS WITH THE FREE RECEPTORS OF THE ORGANISMS AND RABBIT IMMUNE SERUM.

Accordingly, 5 c. c. of the virulent and 5 c. c. of the avirulent prophylactic were each mixed separately with 5 c. c., in dilutions of 1:20, of the same rabbit immune serum employed in the experiments comprising Tables X and XI. After allowing the mixtures to stand for two hours, only a very faint precipitate had taken place, though the fluid above became slightly cloudy in both of the tubes, and more so in the one treated with the avirulent prophylactic than in the other. Prolonged centrifuging did not clear the overlying liquid; and in this condition it was pipetted off from the two tubes and examined separately for its bactericidal properties (see Table XII).

The serum after treatment with the virulent prophylactic showed a value of 0.07 mg. (1:14,000), while that treated with the avirulent one showed a value of 0.04 mg. (1:23,000). The virulent prophylactic had apparently absorbed about $\frac{10}{14}$ of the amboceptors present and the avirulent one somewhat less than $\frac{1}{14}$, a ratio of absorption of about 10:1. However, these results must be regarded with caution, since we were probably encountering here conditions much the same as those seen in the experiments performed with the living organism in the higher dilutions of the serum; that is, the bacteriolytic amboceptors united to the receptors were present in suspension in the slightly

cloudy mixtures. Upon injecting into animals, these amboceptors, after the destruction or solution of the receptors through the aid of the guinea-pig's complement (or of more amboceptors), were not destroyed but again set free, and once more uniting with the receptors of the freshly introduced bacteria evidently gave to the newly added serum an apparently higher bactericidal power. Therefore, the value of the serum after treatment with the virulent prophylactic is probably actually somewhat lower than .07 mg. (1:14,000), and the same may probably be said of the value of .04 mg. (1:23,000) of the serum after treatment with the avirulent strain, although the precipitation of the receptors was probably more complete in this instance than in the case of the virulent prophylactic. Furthermore, in the higher dilutions a smaller number of the combined amboceptors was obviously carried into the animal body. It was impossible to obtain any more accurate results upon the filtration of the fluids, through very dense filters, for, while this process removed the combined amboceptors and receptors, it also removed, as was shown by a control experiment made with the immune serum alone, a considerable number of the free amboceptors. The more concentrated the serum the greater the number of amboceptors removed by filtration. On the other hand, a coarser filtration with filter paper did not separate the combined receptors in suspension. Therefore, this series of experiments suggests forcibly, but does not conclusively demonstrate, that the binding power of the two prophylactics (i.e., of the free receptors) added to an immune serum is within certain limits proportional, first, to the immunity caused by each after its injection into animals (see Tables III–VII), and, second, to the infectious virulence of the respective strain from which it is prepared.

COMPARISON OF THE IMMUNITY OBTAINED WITH THE FREE RECEPTORS OF THE VIRULENT AND AVIRULENT STRAINS.

We may now compare the immunity produced by the injection into rabbits of the free receptors of the virulent cholera organism

with that produced by the injection of those of the avirulent one. These free receptors were obtained, as has already been stated, by the filtration of the killed cholera organisms, which had been subjected to autolytic digestion for varying periods of time in aqueous solutions. These free receptors in the fluid constitute the cholera prophylactic The strength of the prophylactic varies in the different series of experiments according to the number of oesen of the bacteria digested in each cubic centimetre of the fluid. In Prophylactic Number I, 1 c. c. equals the number of receptors obtained by the digestion of one oese of the killed organisms. The strength of the prophylactic in the other series of experiments is indicated in Tables IV–VIII, where the results in immunity are also shown; the tables are self-explanatory. In the animals of Table VII and in a portion of those of Table VIII the inoculations were made subcutaneously, the rabbits comprising the former table receiving injections of the prophylactic in liquid form, and those of the latter the same substance dried in a vacuum and redissolved in saline solution. On comparing the immunity obtained by the injection of the virulent and avirulent receptors from Table III, we see that the ratio of bactericidal immunity between the animals inoculated with the virulent and those inoculated with the avirulent prophylactic varies between 3.5:1 and 12:1. In Table V, the sera obtained from the animals inoculated with the virulent prophylactic showed a bactericidal value of about five and one-half to twelve times that obtained from the injection of corresponding amounts of the "Avirulent," and in Table VI, the animals of the "Virulent" series developed sera having six to fifteen times the value of those of the "Avirulent" one. In Table VII, with subcutaneous inoculation (Nos. 399, 400, 423, and 184), the proportion is from 1:8 to 1:11, and in Table VIII, with the dried prophylactic, the relation is from 1:1.33 to 1:4. The results obtained with the dried prophylactic are certainly not as accurate as those given by the fluid, because of the manipulations to which the powder was subjected, and since they are not in agreement with all of the other numerous experiments in which the liquid prophylactic was

employed, for the purposes of this argument they must be discarded. With this exception, the results here reported with the free receptors are in accord with those which have been obtained by other observers who for inoculation have employed strains of the killed organisms of different virulence; namely, that the immunity obtained is within certain limits approximately proportional to the virulence of the inoculated virus.

COMPARISON OF THE IMMUNITY OBTAINED WITH THE LIVING VIRULENT AND AVIRULENT STRAINS.

We will next turn our attention to the results in immunity obtained by the inoculation of the living virulent and avirulent cholera spirilla. In these experiments the rabbits were given intravenously one-half oese of the living organisms of the respective strains suspended in 1 c. c. of bouillon. Two series of six rabbits each were inoculated, and on the day of the operation in each instance the ratio of virulence between the two strains was verified as 15:1. The results are recorded in detail in Tables I and II, from which we see that by the intravenous injection of the living organisms in quantities of one-half oese the ratio representing the bactericidal value of the sera of the animals inoculated with the virulent and the avirulent organisms was never greater than 4.5:1; i. e., the virulent organism never furnished a serum more than four and one-half times as potent as the avirulent one. Therefore, it cannot be said that the immunity obtained was directly proportional to the virulence of the organisms, since the latter was 15:1 before inoculation. However, with the digested extracts of the organisms of different strains, as we have just seen, and the killed organisms of different degrees of virulence, this may, within certain limits, be said to be the case.

How shall we explain the discrepancy between the virulence of the living organisms and the immunity produced by each? It may be argued that in such a complicated process as immunization the animal cells could not be expected to respond in a proportional manner to such great differences in stimuli, and further that with such doses, as large as one-half oese of the

organisms, we could not expect the immunity to increase proportionally to the virulence, since the animal cells are capable of responding only to a certain limit in the production of immunity, no matter how great the stimulus, and since the number of amboceptors given off becomes proportionally (to a given stimulus) less and less as one approaches this limit. If one is convinced by such an argument, for which it is true there is considerable supporting evidence, no further explanation is necessary. However, on the other hand, it may be seen from the experiments with the intravenous inoculations of the free bacteriolytic receptors from both strains (see Table VI), where the receptors from twelve oesen of the organisms were injected, that the serum of the rabbits inoculated with the receptors from the virulent strain showed a value from six to fifteen times as great as that of those treated with receptors of the avirulent organism.

In these experiments it is to be noted that the stimulus from the receptors of twelve oesen of the virulent strain was equally as great as that from one-half oese of the living virulent organisms, as is evidenced by the fact that about the same bactericidal immunity was obtained in the sera of the animals (comprising Tables I and VI) treated with the virulent strain; however, it must be observed from the experiments of Table V, in which more receptors were evidently obtained upon a more complete digestion of the organisms, and where the stimulus from the amount of receptors was evidently stronger than that from one-half oese of the living virulent organisms, as shown by the value of the sera obtained, the ratio of immunity fell in one instance as low as 5.5:1. Yet, in the other the ratio stood at 12:1. So throughout these experiments (see Tables III to VII), made to determine the comparative value of the sera of more than twenty rabbits, the animals inoculated with the receptors of the virulent strain furnished a serum from three and one-half to fifteen times as strong as that from the animals inoculated with a corresponding amount of those from the avirulent one. However, in only one instance was the low ratio of 3.5:1 obtained, the next lowest being 5.5:1, and the next 6:1 and 7:1.

Likewise, turning to results other than our own, we see that Ascher (20) found, upon the intravenous injection of varying amounts of the killed cholera spirillum into rabbits one oese gave rise to a bactericidal immunity more than thirty times as great as one produced with one-tenth or two-tenths oese. He also observed that, while two and one-half oesen in two cases gave less than twice as great a bactericidal immunity as one oese, ten oesen produced a serum of ten times the bactericidal power of the one produced by one oese. Therefore, while evidently the individuality of the animal is an important factor in the degree of the immunity produced, a fact borne out by the varying results seen in our tables, and while also it is evident that with very large doses the immunity is not directly proportional to the quantity of the organism inoculated, at least for amounts of one-half oese (reasoning from the immunity obtained with the free bacteriolytic receptors and that with the killed organisms), the explanation of the difference of immunity of 4:1 as against that virulence of 15:1 is still lacking. At least no explanation given previously would seem to be satisfactory, whether it be solely upon the ground that the cells in the case of the animal inoculated with the virulent strain have already produced the maximum amount of amboceptors of which they are capable, or upon the ground that their limit of production of amboceptors is so nearly reached by a stimulus resulting from a considerably smaller number of receptors than one-half oese of the virulent organisms furnishes that the increased stimulus produced by this amount of the virulent organism gives rise to so small an increase in immunity that its ratio to that produced by one-half oese of the avirulent strain is never greater than 4:1.

Therefore, while it is admitted that we should not necessarily always expect in our animals an immunity fifteen times as great from the injection of a stimulus fifteen times as powerful, we might anticipate, if we reason from the results obtained by the injection of the killed organisms or their extracts, that a ratio of immunity nearer to 15:1 than that of 2.5:1 to 4.5:1 would be obtained, when amounts not larger than one-half oese

of the living organisms are injected. Hence, it would seem necessary to seek for some other explanation for these results. The idea is suggested that something has happened to the living avirulent strain after its injection into the animal which increases its virulence and brings it into greater agreement with the virulent one, so that the ratio of 15 : 1 is lessened, the dissimilarity being existent at the moment preceding injection, as is evidenced by the fact that the virulent organism will kill in doses one-fifteenth as great as the avirulent. Should the different strains be killed at this moment and injected, or killed and digested and then injected, this change does not take place. The ratio of 15 : 1, as evidenced by the immunity obtained, is within certain proportions retained; hence, depriving the avirulent strain of its life would seem to be at least one of the factors which prevented this change in the ratio.

Let us now consider the influence which an animal succumbing to an infection with a given bacterium has exerted upon the infecting organism during the period of its parasitic life upon the host, and also the influence which a normal immune serum exerts upon the virulence of a bacterium which has been cultivated in it.

Since the classical observations of Pasteur and his pupils in 1881 (21), we have known that in general attenuated races of bacteria can be re-endowed with lethal properties by successive passages through susceptible animals. Indeed, this is the method usually employed for increasing the virulence of a given bacterium. However, there is a limit to this with every organism, and a culture which had attained its highest possible virulence was designated by Pasteur as a "fixed virus" (22).

We also know from many observations that the virulence of an organism may be greatly increased by its repeated inoculation into fresh serum. Thus, Roger (23) as early as 1889 reported that streptococci which through cultivation in bouillon had lost most of their energy of growth and virulence would regain these powers when they were repeatedly inoculated in rabbit serum.

Trommsdorff (24) also found that organisms which had been

grown in fresh serum showed an increased resistance to bacteriolysis. Danysz (25) maintained that anthrax bacilli, when inoculated into fresh serum, became surrounded with a sort of mucous covering, which latter protected them to a certain extent against the action of bactericidal serum.

Metchnikoff and Roux (26) have shown that the virulence of an organism may be greatly increased by growing it in collodion sacs within the abdominal cavity of an animal.

Professor Welch (27), in his Huxley lecture on recent studies in immunity, in advancing an hypothesis by which might be explained the source, the mode of production, and the nature of certain bacterial toxins, pointed out that "certain substances in the host of cellular origin assimilable by the parasites through the possession of haptophore groups with the proper affinities become anchored to the receptors of the parasitic cell, which, if not too much damaged, is thereby stimulated to the overproduction of like receptors. These excessive receptors of the parasite, if cast into the fluids or cells of the host, are constituted intermediary bodies or amboceptors with special affinities for these cellular constituents or derivatives of the host, which may lead to their production and for which they possess in whole or in part identical receptors. Provided the host is supplied also with its appropriate complements, there result cytotoxins with special affinities for certain definite cells or substances of cellular origin in the host. The contribution of the parasitic cell to these cytotoxins is the amboceptors; either the parasite or the host may provide the complements."

In considering the condition of the bacterium as well as that of the animal host, according to the hypothesis advanced, the struggle between the bacteria and the body cells in infections may be conceived as an immunizing contest, in which each participant is stimulated by its opponent to the production of cytotoxins hostile to the other, and thereby endeavors to make itself immune against its antagonist.

Ainley Walker (28) performed a series of experiments, which, so far as they went, supported this hypothesis of Professor Welch. Walker showed by growing typhoid bacilli in

bouillon to which were added increasing amounts of immune serum (free from the complement), that their virulence and resistance to serum were increased and their agglutinability diminished. In another series of experiments (29), he found that the progressive passage of typhoid bacilli through fresh bacteriolytic normal rabbit serum mixed with bouillon in the proportion of 1 : 10 produced a distinct increase in the virulence of the bacilli towards rabbits and guinea-pigs, and also increased their resistance to bacteriolytic serum, as shown by the plate culture method.

Welch's hypothesis includes the explanation which Walker gives for the result of his experiments, and also more. According to the idea of the former, certain bacterial anti-bodies (discharged receptors) are capable, not only of neutralizing the immune bodies of the host, but with aid of the complements also of poisoning the cells of the latter.

Keeping these ideas in view, let us attempt next to trace the biology of an avirulent strain from the moment of its intravenous injection into a non-immunized rabbit. Upon the arrival of the organisms in the blood stream they quickly disappear and indeed are, we suppose, soon killed, though just how rapidly we do not know. Pfeiffer and Marx (30) assumed that in a short time the cholera spirilla became anchored to the cells of the spleen, the bone marrow, and the lymph glands, since it was in these organs that the specific protective substances were particularly formed. However, in a later series of experiments Pfeiffer (31) was not able to demonstrate conclusively this increased anchoring power for cholera spirilla of the cells of the spleen.

It would seem to be a mistaken idea to suppose that the immediate destruction of the organisms is in all cases inevitable, since we know, for example, from the injection of avirulent strains of Micrococcus melitensis into the blood current of monkeys, that the organisms may remain alive for a period of time and even be recoverable in cultures. Yet eventually they disappear and the recovery of the animal results.

We also know that in many infectious diseases (typhoid fever, etc.) the organisms may be obtained by culture from the

circulating blood and yet finally these bacteria become destroyed and the patient survives the malady.

The most important results, in this connection, which have been obtained with the cholera spirilla are those of Kolle (32). He found that, upon injection of one-half oese of the living cholera organisms into the carotid of guinea-pigs, blood drawn at intervals of from five to fifteen minutes after the injection by streak cultures upon an agar plate showed only a few and widely scattered colonies. Evidently within this time the majority of the organisms had been destroyed, although some were still alive. In these experiments and at the same period of time, the bacteria were found to be no more numerous in the spleen than in the blood current. From the observation of Pfeiffer's phenomenon in the abdominal cavity of guinea-pigs, we also know that in some cases (even when the animal eventually recovers) some of the organisms may remain alive for more than one-half hour after their injection, and it is only later that they become disintegrated.

For the moment, then, let us suppose that our organism is of a sufficiently great virulence to be capable of surviving for at least a few generations, and that, while the animal becomes very ill after inoculation, it eventually recovers. These successive generations of the bacterium multiplying in the rabbit serum will, we suppose, rapidly increase in virulence, i.e., their haptophore groups will rapidly rise in number, owing to the stimulus received from the occupation of the receptors of the bacteria by the amboceptors of the normal serum. (Compare with Walker's results with typhoid bacilli in fresh normal serum.) That is, reasoning from the well-known biological law of Weigert, an injury to the bacterial cells will be produced and an excessive generation of the receptors result. Hence, the final result in immunity in this instance will be much greater than it would if the successive generations became no richer in receptors than the one existing at the time of inoculation.

On the other hand, let us trace the fate of the virulent organism upon its injection into the circulation. This strain has already reached its maximum virulence and become a "fixed virus," i.e., it is already saturated with haptophore groups. Hence,

its few successive generations can become no richer in such groups than the one used for the inoculation; so that the immunity produced can only correspond to, or at least only equal, that which would result from the generation existing at the time of inoculation, multiplied by the number of generations for which the organism survives. Therefore, the immunity obtained by the organism of maximum virulence would not be so great, compared with the stimulus, as would that produced in the case of the living avirulent germ. Furthermore, is it not conceivable that, if the same stimulus were received by the virulent organism as by the avirulent one, the latter, which is so poor in receptors, would feel the injury more severely than the former, which is so well protected and so rich in these bodies. Hence, would not the regeneration, provided an immediate destruction of the organism did not occur, eventually be greater in the case of the avirulent strain? We know that the number of receptors in the virulent organism must be enormous. We can conceive that it may possess many more receptors than would be required to bind all the existing amboceptors in the normal serum. Hence, if there is still an excess of unbound receptors, will this organism be stimulated as strongly to the generation of others as the avirulent one, in which no such excess exists?

Such theoretical conceptions are difficult to confirm. In the first place, it does not seem likely that even one division of the cholera spirillum would take place after its inoculation into the blood circulation, since the shortest period within which this organism has been known to divide, at least on artificial media, is about nineteen minutes. We have no observations to show that this phenomenon takes place in a very much shorter time in the animal body. Provided the organisms were just about to divide at the moment following their inoculation, it is questionable whether any of them would be alive to undergo the same process again at the end of nineteen minutes; but, as already stated, we do not know the exact period at the end of which all the organisms will have perished. Ordinarily, as we have seen, after an injection into the circulation of rabbits of a small amount of the cholera spirilla, the organisms after a few minutes

can be obtained, if at all, only in slight numbers, from small quantities of the blood. This, however, does not necessarily mean that all of the spirilla in the animal body have been destroyed within this period. On the other hand, it would seem probable that the increase in virulence in the avirulent organism would begin at the moment of the inoculation. Theoretically, therefore, in the brief period of time preceding its destruction it would have an opportunity of increasing its haptophore groups and becoming more like the virulent strain. Still, whether the explanation outlined above is the correct one for this phenomenon can not be conclusively demonstrated without further experimental work, and we must admit that we are at present unable, in an entirely satisfactory manner, to account for such a small variation in immunity after the employment of two strains of such different degrees of virulence. Perhaps additional light may be thrown upon the solution of this problem by the performance of similar experiments with other micro-organisms than the cholera spirillum.

It seems evident that Professor Welch's hypothesis is very applicable to the cholera organism in its relation to infection and immunity, and explains the reason why, as we have seen, it is only with great difficulty that we are able to obtain even small amounts of intracellular toxin from our cultures on artificial media. It further explains how, in the animal body (particularly in the mucosa of the human intestine) the organisms, by the binding of suitable amboceptors to their own receptors, are capable of becoming much richer in endo-toxin and indeed of generating a considerable excess of it within a very brief period. Such a process applies also in the immunization of animals with the living organism, though, from the observations made on the injection of the cholera spirilla into the blood circulation of animals, it would seem that the bacteria do not find in the blood stream, etc., the same favorable stimulus for the production of the toxic receptors as they do in the mucosa of the human intestine. On the other hand, the proper amboceptors for the production of the bactericidal and agglutinative substances are here encountered. This conception also explains the difficulty

which we have experienced in obtaining large amounts of cholera anti-toxin, and the relative ease with which bactericidal substances are produced in the serum of the inoculated animals.

It would seem that the living avirulent strain by some process (not as yet satisfactorily explained in its entirety) is capable of increasing the number of its haptophore groups in the animal body after its injection into the circulation and before its total destruction, so that a relatively higher immunity is obtained by it than is produced when an organism of maximum virulence is employed. In other words, while in the case of the living organisms a greater immunity is to be obtained from the more virulent strain, such immunity is not necessarily in direct proportion to the virulence of the bacteria employed, as is the case, within certain limits, with the killed bacteria or with their free receptors.

CONCLUSIONS.

The virulent cholera spirillum possesses a greater number of bacteriolytic and agglutinable haptophore groups, or these groups are endowed with a greater avidity for uni- and amboceptors than the avirulent.

The number or the avidity of the bacteriolytic receptors possessed by a bacterium is directly proportional to its virulence.

However, the agglutinable receptors do not follow this law, i.e., the agglutinable haptophore groups are not necessarily present in the same proportion as the bactericidal ones.

While the energy of growth is a very important factor in relation to virulence, other phenomena must also be considered.

The virulent organism possesses a greater number of toxic haptophore groups than the avirulent.

The binding power of the free receptors of the organisms for bacteriolytic amboceptors in vitro is proportional to the bactericidal immunity produced in animals by each, which latter is in turn proportional to the virulence of the organisms from which the receptors were extracted. The binding power in vitro of the dead micro-organisms of different virulence for bacteriolytic amboceptors is not in proportion to their toxicity.

The bactericidal immunity obtained by means of the inoculation with the dead organisms of different virulence, or their extracts obtained by autolytic digestion, is proportional to the virulence of the living strains of the bacteria employed.

With the living organisms, while the bactericidal immunity obtained from the inoculation of animals with the virulent organism is greater than that produced with the avirulent, such immunity is not in direct proportion to the virulence of the bacteria introduced.

These conclusions apply to the two strains of cholera spirilla employed in the foregoing experiments. Whether they will also hold good with other strains of this spirillum or for microorganisms in general, must be decided by further experimental work.

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TABLE No. I.

The average weight of rabbits, 1500 grams.
The injections were made into an ear vein with ½ oese of the living organisms suspended in 1 c. c. bouillon. All animals were killed by bleeding one week after inoculation. Agglutination experiments performed with both stems, "virulent" and "avirulent." Bactericidal reactions performed only with the "virulent" stem.

Rabbit No.	Inoculated with ½ Oese intraven.	Agglutination Experiments. Organism	Dilution of serum. 1:50 1:100 1:200 1:300 1:400 1:500 1:600 1:700 1:800	Controls NaCl. Sol.	Controls NaCl. no serum	Bactericidal reactions (Pfeiffer's Phenomenon.) Dilution of serum. 1:50 1:100 1:200 1:300 1:400 1:500 1:600 1:700 1:800 1:900 1:1000 1:2000 1:4000 1:6000 1:8000 1:10000 1:12000 1:14000 1:15000	Control Animals Without Serum.
V.	"Virulent"	"virulent"	W W N N N N N	N	N	A A A A A A A A A A A A A A A A A A N/D	6 control animals all neg; all dead in 24 hours.
		"avirulent"	N N	N	N		
VI.	"Virulent"	"virulent"	W W N N N N	N	N	A A A ± A A A A A A A A A A A A A A A A A A ± A A A A A A A A A A A A A A A A A A N/D	9 control animals all neg; all dead in 24 hours.
		"avirulent"	W N	N	N		
VII.	"Virulent"	Died after five days. Agar plate cultures from all organs were sterile					
VIII.	"Avirulent"	"virulent"	W W N N N N N	N	N	A A A A A A A A A A A A A A A A A A ± A N/D	5 control animals all neg; all dead in 24 hours.
		"avirulent"	W W N N	N	N		
IX.	"Avirulent"	"virulent"	W W N N N N N	N	N	A A A A A A A A A A A A A A A A A N/D	8 control animals all neg; all dead in 24 hours.
		"avirulent"	W N	N	N		
X.	"Avirulent"	"virulent"	W N N N N N N	N	N	A A A A A A A A A A A A A A A A A N/D	7 control animals all neg; all dead in 24 hours.
		"avirulent"	W N N	N	N		

= Complete agglutination. Overlying liquid clear.

= Distinct agglutination with precipitation. Overlying liquid not entirely clear.

A = Microscopically, positive bactericidal reaction. Animal alive after 24 hours.

N/D = Microscopically, negative bactericidal reaction. Animal dead after 24 hours.

±/A = Microscopically, Pfeiffer's Phenomenon doubtful; Animal alive after 24 hours.

TABLE No. II.

Inoculations with living organisms
average weight of rabbits 1150 grams.
(For explanation see Table No. 1)

Rabbit No	Inoculated with ½ Oese intraven.	Agglutination Experiments. Organism	Dilution of Serum. 1:50 1:100 1:200 1:300 1:400 1:500 1:600 1:700 1:800 1:900 1000	Controls No. Cl. Sol; no serum	Bactericidal reactions (Pfeiffer's Phenomenon). Dilution of Serum. 1:100 1:1000 1:2000 1:3000 1:4000 1:5000 1:6000 1:8000 1:9000 1:10000 1:15000 1:16000 1:17000 1:18000 1:19000	Control Animals Without Serum.
194	Virulent	"virulent"	N N N	N N	N	2 control animals; reactions neg; all dead in 24 hours.
		avirulent	W N N	N N	A A A A A A D	
195	Virulent	"virulent"	W	N N	N	1 control animal; reaction neg; dead in 24 hours.
		avirulent	W	N N	A A A A A ± N D	
196	Virulent	"virulent"	N N	N N	N	2 control animals; reactions neg, all dead in 24 hours.
		avirulent	W N	N N	A A A ± N A A A D	
197	Avirulent	"virulent"	N N	N N	N	2 control animals; reactions neg; all dead in 24 hours.
		avirulent	N N	N N	A A A A A A A A N D	
198	Avirulent	"virulent"	N N	N N	N	2 control animals; reactions neg; all dead in 24 hours.
		avirulent	W N	N N	A A A A A A A ± D	
199	Avirulent	"virulent"	N	N N	N	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	N	N N	A A A A A N D	

TABLE No. III.

Inoculations with "Prophylactic"
(For explanation see Table No. I.)

Rabbit No.	Inoculated with 1 C.C. intraven.	Agglutination Experiments. Organism	Dilution of Serum. 1-50 1-100 1-200 1-300 1-400 1-500 1-600 1-700	Controls Na Cl sol; no serum.	Bactericidal reactions (Pfeiffer's Phenomenon). Dilution of Serum. 1-50 1-100 1-200 1-300 1-400 1-500 1-600 1-700 1-800 1-900 1-1000 1-2000 1-3000 1-4000 1-5000 1-8000 1-10000 1-12500	Control Animals Without Serum.
58	"Virulent"	"virulent"	N N N N	N N	A A A A A A A D	5 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I.	"avirulent"	W W N N	N N		
59	"Virulent"	"virulent"	W N N N	N N	A A A A A A D	4 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I.	"avirulent"	W W N N	N N		
60	"Virulent"	"virulent"	N N N	N N	A A A A A A + D	3 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I.	"avirulent"	W W N	N N		
61	"Avirulent"	"virulent"	W N N N	N N	A A A A A A N D N D	4 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I.	"avirulent"	N N N	N N		
62	"Avirulent"	"virulent"	N N N N	N N	A A A A A A A A + N D	3 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I.	"avirulent"	W W N N N	N N		
63	"Avirulent"	"virulent"	N N N N	N N	A A A A A A A A N D	3 control animals; reactions neg., all dead in 24 hours.
	Prophylactic I.	"avirulent"	N N N N	N N		

TABLE No. IV.

Inoculations with "Prophylactic II."
(For explanation see Table No. I.)

Rabbit No.	Inoculated with 12 C.C. intraven. (1 C.C. = 1 oese)	Agglutination Experiments. Organism	Dilution of Serum. 1-50 1-100 1-200 1-400 1-600 1-700 1-800 1-900	Controls Na Cl Sol; no serum.	Bactericidal Reactions (Pfeiffer's Phenomenon). Dilution of Serum. 1-100 1000 5000 10000 15000 17000 18000 19000 20000 22000 23000	Control Animals Without Serum.
86	"Virulent" Prophylactic II. digested 2 days.	"virulent"	W N N	N N	A A A A A A A N D	2 control animals; reactions neg.; all dead in 24 hours.
		avirulent	W N	N N		
87	"Virulent" Prophylactic II. digested 2 days.	"virulent"	W N N	N N	A A A A A A N D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	N N	N N		
88	"Virulent" Prophylactic II. digested 5 days.	"virulent"	IV N	N N	A A A A A A N D	2 control animals; reactions neg.; all dead in 24 hours
		avirulent	W	N N		
89	"Virulent" Prophylactic II. digested 5 days. & reheated 2 hrs. 60°C	"virulent"	W N N N	N N	A A A A A + N D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	W N N N	N N		

TABLE No. V.

Inoculations with "Prophylactic III."
digested for five days.
(For explanation see Table No. I.)

Rabbit No.	Inoculated with: 6 c.c. intraven. (1 c.c. = 2 oese.)	Agglutination Experiments: Organism	Dilution of Serum: 1-50	1-100	1-200	1-300	1-400	1-500	1-600	1-700	1-800	1-900	1-1000	1-2000	Controls Na. Cl. Sol. no Serum		Bactericidal Reactions (Pfeiffer's Phenomenon) Dilution of Serum: 1-100	1-500	1-1000	2000	4000	5000	6000	10000	15000	20000	22000	24000	25000	Control Animals Without Serum.
192	"Virulent" Prophylactic III.	"virulent"											W	N	N	N													6 control animals; reactions neg; all dead in 24 hours.
		"avirulent"										W	W	N	N	N													
193	"Virulent" Prophylactic III.	"virulent"											W	N	N	N													5 control animals; reactions neg; all dead in 24 hours.
		"avirulent"											W	N	N	N													
204	"Avirulent" Prophylactic III.	"virulent"				W	N	N	N	N						N	N													5 control animals; reactions neg; all dead in 24 hours.
		"avirulent"						W	N	N						N	N												
205	"Avirulent" Prophylactic III.	"virulent"					W		N	N	N					N	N													4 control animals; reactions neg; all dead in 24 hours.
		"avirulent"							W	W	N					N	N												

TABLE No. VI.

Inoculations with "Prophylactic IV."
digested for 2 days.
(For explanation see Table No. I.)

Rabbit No.	Inoculated with: 12 c.c. intraven. 1 c.c. = 1 oese.	Agglutination Experiments. Organism	Dilution of Serum: 1-100	1-200	1-300	1-400	1-500	1-600	1-700	1-800	1-900	Controls Na. Cl. Sol. no Serum.		Bactericidal Reactions (Pfeiffer's Phenomenon) Dilution of Serum: 1-500	1000	2000	4000	5000	10000	12000	13000	15000	16000	Control Animals Without Serum.
256	"Virulent" Prophylactic IV.	"virulent"							W	N	N	N	N											1 control animal; reaction neg., dead in 24 hours.
		"avirulent"								N	N	N	N											
257	"Virulent" Prophylactic IV.	"virulent"							N	N	N	N	N											1 control animal; reaction neg.; dead in 24 hours.
		"avirulent"							N	N	N	N	N											
177	"Avirulent" Prophylactic IV.	"virulent"				N	N					N	N											1 control animal; reaction negative; dead within twenty four hours.
		"avirulent"					N					N	N											
178	"Avirulent" Prophylactic IV.	"virulent"			W	N	N					N	N											
		"avirulent"				W	N					N	N											

TABLE No. VII.

Subcutaneous Inoculations with Prophylactic.
(For explanation see Table No. I.)

Rabbit No.	Inoculated Subcutaneously with.	Agglutination Experiments: Organism	Agglutination Experiments: Dilution of Serum: 1-20 1-50 1-100 1-200 1-300 1-400 1-500 1-600 1-700	Controls Ka.Cl. Sol.; no Serum	Bactericidal Reactions (Pfeiffer's Phenomenon:) Dilution of Serum. 1-100 1-200 1-300 1-400 1-500 1-700 1-900 1-1000 2000 4000 5,000 6,000 7000 8,000 9,000 10,000 12,000	Control Animals Without Serum.
399	5 c.c. Virulent Prophylactic V. 1 c.c. = 8 oese	virulent	W W N	N N	A A A A A A N/D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	N	N N		
400	5 c.c. Virulent Prophylactic V.	virulent	W W W N	N N	A A A A ± N/A D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	W N	N N		
423	5 c.c. Virulent Prophylactic V. Preserved in 5% carbolic acid	virulent	W W W N	N N	A A A N/A D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	W W N	N N		
426	2 c.c. Virulent Prophylactic V. repeated to 60° C. for 30 min.	virulent	N N	N N	A A A N/A D	1 control animal, reaction neg., dead in 24 hours.
		avirulent	N N	N N		
174	5 c.c. Avirulent. Prophylactic IV 1 c.c. = 1 oese.	virulent	N N	N N	A A ± N/A A D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	N N	N N		
184	5 c.c. Avirulent Prophylactic V.	virulent	W N	N N	A A A A A ± N/A D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	N	N N		
440	5 c.c. Virulent Prophylactic VI. in Chlorotone (1 c.c. = 8 oese)	virulent	W N N N	N N'		
		avirulent				

TABLE No. VIII.

Inoculations with Dried Prophylactic redisolved in Na. Cl. sol.
(For explanation see Table No. I.)

Rabbit No.	Inoculated with	Agglutination Experiments. Organism	Dilution of Serum. 1-20	1-40	1-50	1-60	1-80	1-100	1-200	Controls Na.Cl. sol; no Serum.		Bactericidal Reactions (Pfeiffer's Phenomenon) Dilution of Serum. 1-50	1-100	1-200	1-300	1-400	1-500	1-600	1-700	1-800	1-1000	1-2000	1-3000	1-4000	1-5000	Control Animals Without Serum.
167	10 Mgs. Virulent II.	"virulent"							N	N	N															2 control animals; reactions neg.; all dead in 24 hours.
		"avirulent"							N	N	N	A	A	A		A	A		A	A	A	A	A	A	D	
168	3 Mgs. Virulent II.	"virulent"				N	N			N	N						N/									2 control animals; reactions neg.; all dead in 24 hours.
		"avirulent"				W	N			N	N	A	A	A	A	A	D									
187	(Subcutaneous) 5 Mgs. Virulent III.	"virulent"						N	N	N	N								N/							2 control animals; reactions neg.; all dead in 24 hours.
		"avirulent"						W	N	N	N	A	A	A	A	A			D							
169	10 Mgs. Avirulent II.	"virulent"						N	N	N	N											N/				2 control animals; reactions neg.; all dead in 24 hours.
		"avirulent"						W	N	N	N	A	A	A	A		A		A	A	D					
170	3 Mgs. Avirulent II.	"virulent"			N					N	N					N/										1 control animal; reaction neg.; dead in 24 hours.
		"avirulent"			N					N	N	A	A	A	A	D										
175	(Subcutaneous) 5 Mgs. Avirulent III.	"virulent"					N	N		N	N						N/									1 control animal; reaction neg.; dead in 24 hours.
		"avirulent"		W			N	N		N	N	A	A	A	A	A	D									

TABLE No. IX.

Showing Bactericidal value of Guinea pig serum after treatment for 2 hrs. with the virulent and avirulent strain.

Original Bactericidal value of Serum. { *1-9,000 Post. Alive* / *1-10,000 Neg. Dead.*

Original Dilution of Serum.	*5 c.c. of Serum Centrifuged with*	*Bactericidal Reactions (Pfeiffer's Phenomenon) Actual dilution of Serum.* 1:100 · 1:200 · 1:300 · 1:400 · 1:500 · 1:600 · 1:700 · 1,000 · 2,000 · 5,000 · 6,000 · 6,500 · 7,000 · 8,000 · 8,500 · 9,000 · 10,000	*Control Animals Without Serum.*
1-20	*5 Oese Virulent*	A A A A A N/D N/D	*1 control animal; reaction neg.; dead within 24 hrs.*
1-20	*5 Oese Avirulent*	A A N/D N/D	*1 control animal, reaction neg.; dead within 24 hrs*
1-100	*5 Oese Virulent*	A A A A N/D	*1 control animal; reaction neg.; dead within 24 hrs.*
1-100	*5 Oese Avirulent*	A A A N/D	*1 control animal; reaction neg.; dead within 24 hrs.*

TABLE No. X.

Showing Bactericidal value of Rabbits serum after treatment for 2 hrs. with the virulent and avirulent strain.
Original Bactericidal value of serum... { *1-24,000 Post. Alwe* / *1-25,000 Neg. Dead*

Original Dilution of Serum	*5 c.c. Serum Centrifuged with*	*Bactericidal Reactions (Pfeiffer's Phenomenon) Actual dilution of Serum.* 1-80 · 1-160 · 1-300 · 1-700 · 1-800 · 1-900 · 1,000 · 1,100 · 1,200 · 1,400 · 2,400 · 2,500 · 2,800 · 4,000 · 5,000 · 6,000 · 8,000 · 10,000 · 15,000 · 20,000 · 22,000 · 22,400 · 22,500 · 23,000 · 24,000
1-20	*5 Oese Virulent*	A A A A A A + A N/D N/D — *1 Control animal, without serum reaction neg., Dead within 24 hours.*
1-20	*5 Oese Avirulent*	A A A — *1 Control animal Dead within 24 hrs.* — A A — A — A — A A A + + N/D — N/D
1-100	*5 Oese Virulent*	A A A A A N/D — *1 Control animal Dead within 24 hours.*
1-100	*5 Oese Avirulent*	*1 Control animal dead within 24 hours.* — A A — A A A A A A N/D
1-500	*5 Oese Virulent*	*1 Control animal dead within 24 hours.* — A A A A N/D N/D
1-500	*5 Oese Avirulent*	*1 Control animal dead within 24 hours.* — A — A A N/D N/D

TABLE No. XI.

Showing Bactericidal value of Rabbit serum after treatment for 2 hrs. with the killed virulent & avirulent organisms.
Original Bactericidal value of serum. { *1-24,000 Post. Alive.* / *1-25,000 Neg. Dead.*

Original Dilution of serum + bouillon.	*5 c.c. Serum. Centrifuged with*	*Bactericidal Reactions (Pfeiffer's Phenomenon.) Actual dilution of serum.* 1-200	1-400	1-800	1-1,000	2,000	3,000	4,000	5,000	6,000	10,000	15,000	20,000	21,000	22,000	24,000	*Control Animals Without Serum.*
1-40	5 Oese Killed Virulent in bouillon	A	A	A	A	A	A	A	A	N / D							1 Control; reaction neg.; dead within 24 hours.
1-40	5 Oese Killed Avirulent in bouillon	A	A		A				A		A	A	A	A	± A	N / D	1 Control; reaction neg.; dead within 24 hours.

TABLE No. XII.

Showing Bactericidal value of Rabbit serum after treatment for 2 hrs with virulent & avirulent Prophylactic IV.
Original Bactericidal value of serum. { *1-24,000 Post. Alive* / *1-25,000 Neg. Dead.*

Original Dilution of serum + Prophylactic	*5 c.c. of Serum. Centrifuged with*	*Bactericidal Reactions (Pfeiffer's Phenomenon.) Actual dilution of Serum.* 1-100	1-1,000	1-5,000	1-8,000	1-10,000	12,000	14,000	16,000	20,000	23,000	24,000	*Control Animals Without Serum.*
1-40	5 c.c. 'Virulent' Prophylactic IV.	A	A	A			A	A	N / D				1 Control; reaction neg.; dead within 24 hours.
1-40	5 c.c. 'Avirulent' Prophylactic 'IV.			A	A					A	A	N / D	1 Control; reaction neg.; dead within 24 hours.

A METHOD FOR PREPARING A PERMANENT NOCHT'S STAIN (NOCHT-JENNER STAIN).

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PLATES XXI. AND XXII.

The possibility of preparing a permanent staining solution from the formulas for Nocht's (1) stain was suggested by the constant use of Jenner's (2) stain for routine blood examinations and malarial staining during the summer and fall months of 1901. In malarial blood the pigmented bodies of tertian fever and the crescent bodies of æstivo-autumnal fever are readily demonstrated with Jenner's stain, and the use of polychrome stains was resorted to only in doubtful cases showing no full-grown forms of parasites.

Goldhorn's (3) method of staining with the polychrome blue prepared from methylene blue and lithium carbonate proved valuable, but we were never able to obtain the constantly perfect results obtained by employing the methods of Nocht as described by Lazier (4) and Ewing (5). No other method gives results comparable to these. The only objection to them is the time required (from two to twelve hours) for good staining.

During the fall of 1901, repeated attempts to prepare a permanent staining fluid by Nocht's method succeeded in so far that in the later preparations the same method of procedure was followed, but we were unable to obtain the polychrome tint for the nuclear material and the chromatin staining in the malarial parasite until the publication of Leishman (6) suggested the final simple step in staining, that of diluting the staining fluid with distilled water after staining one minute in full strength and allowing this diluted stain to remain in contact with the specimen from three to five minutes.

The staining fluid consists of a powder, prepared by mixing solutions of eosin and polychrome blue and Ehrlich's rectified methylene blue, dissolved in pure methylic alcohol, and the methods of preparation and of staining combine, therefore, the principles of Nocht, Jenner, and Leishman.

The methods described by May and Grünwald (7), Michaelis (8), Reuter (9), Willebrand (10), Wright (11), and Schegoleff (12) resemble Jenner's and Leishman's, and none of them gives the intense clear staining obtained by employing Nocht's (13) principle of mixing three solutions.

Nocht (14) pointed out that the essential staining element of the Romanowski (15) and Ziemann (16) methods is a new material which he designated as "red from methylene blue" (Michaelis's "methylen-azur," or "azurblau" (17)), which is formed in all alkaline methylene-blue solutions.

Nocht's first (1899) alkaline methylene blue consisted of a 1% aqueous solution of methylene blue with 0.5% soda, which stood for a few days at a temperature of from 50°–60° C.

A few months later Nocht (18) reported a method for making the polychrome methylene blue (alkaline), by steaming the 1% methylene blue and 0.5% soda mixture in a stew-pot for a few hours, filtering and neutralizing. Thereafter this solution was added to the original 1% aqueous methylene-blue solution, and later (1903) Nocht (19) described a third method for the preparation of polychrome methylene blue. To 100 c. c. of 1% methylene blue solution was added the silver oxide precipitated from one gram of silver nitrate in solution by the addition of alkali in sufficient quantity, and this mixture was allowed to digest four or five days at room-temperature. This last method has not furnished us a good chromatin-dye, nor can it be used for preparing the stain to be described. Nocht's second method is used. The polychrome borax-methylene blue of Ziemann (20) was found unsatisfactory.

PREPARATION OF THE STAINING FLUID.

Two stain powders are required: the water-soluble, yellow eosin (Grübler) and Ehrlich's rectified methylene blue (Grübler).

The polychrome blue is readily prepared from the latter after the following directions:

Methylene blue (Ehrlich's rectified).....2 grm.
Sodium carbonate (dry powder).....2 grm.
Distilled water .....200 c. c.

Dissolve the sodium carbonate in hot distilled water and stir in the methylene blue powder in the above proportions; bring the mixture to a moderate boil in an evaporating dish on a wire gauze over the flame, or heat over a boiling water-bath, for from ten to fifteen minutes; add from 30 to 40 c. c. of distilled water for each 100 c. c. of solution (i.e., from 60 to 80 c. c.) to replace water lost by evaporation, and heat for from ten to fifteen minutes longer. The hot solution is poured off from the sediment and made up to 200 c. c. with distilled water if necessary. This should be partially neutralized before further mixing, by the addition of from 12.5% to 20% acetic acid. It is well to add the acetic acid to one-half the polychrome-blue solution until a well-marked acid reaction to litmus is obtained (6 or 7 c. c. of 12.5% acid or 3 or 4 c. c. of 20% acid to 100 c. c.), and to mix this neutralized portion with the un-neutralized half, thus preventing over-neutralization. The solution should be alkaline in final reaction since a slight excess of acid destroys the polychrome properties which cannot be restored by addition of alkalies.

A guide to successful preparation is the mixing of the solutions according to Ewing's directions:

A. =	Distilled water.....	10 c. c.
B. =	1 % aqueous eosin.....	3-5 drops
C. =	Freshly prepared polychrome-blue solution.....	6 drops
D. =	1 % aqueous methylene blue.....	2 drops

If the specimens stained in the above mixture give good results in from two to twelve hours, the polychrome-blue solution may be used for preparing the powder.

From Ewing's directions the following proportions for 1000 c. c. of water were calculated, taking forty-five drops equivalent to one fluid dram and to

3.7 c. c. Thus for 10 c. c. of water, the equivalents for the same solutions would be:

B.	3 drops = 0.246 c. c., approximately	0.25 c. c.
C.	6 " = 0.492 c. c. "	0.50 c. c.
D.	2 " = 0.164 c. c. "	0.16 c. c.

and for 1000 c. c. of water:

B.	24.6 approximately	25 c. c.
C.	49.2 "	50 c. c.
D.	16.4 "	16.5 c. c.

These proportions give a comparatively minute quantity of precipitate, and the final product does not stain well.

The following mixtures in 10 c. c. of water were made, always taking B. in quantity approximately equal to half of C., and D. in quantity equal to a third of C.

1.	A. = Distilled water	10 c. c.
	B. = 1 % aqueous eosin	5 c. c.
	C. = Polychrome blue	10 c. c.
	D. = 1 % aqueous methylene blue	3.5 c. c.
2.	A.	10 c. c.
	B.	3 c. c.
	C.	6 c. c.
	D.	2 c. c.
3.	A.	10 c. c.
	B.	1.5 c. c.
	C.	3 c. c.
	D.	2 c. c.
4.	A.	10 c. c.
	B.	1 c. c.
	C.	2 c. c.
	D.	0.66 c. c.

Mixtures *3* and *4* gave good results, but when mixed in proportion corresponding to 1000 c. c. of water, the maximum amount of precipitate with good staining was obtained by using from 70 to 80 c. c. of D (aqueous methylene blue 1 %) in No. *4*—that is:

5.	A. Distilled water	1000 c. c.
	B. 1 % aqueous solution eosin	100 c. c.
	C. Polychrome-blue sol	200 c. c.
	D. 1 % aqueous methylene-blue sol	70 c. c.

Twenty-one mixtures were prepared in proportions of No. *5*, and tested as to staining properties, and one with B, C, and D, in proportion without distilled water. The variations in these mixtures consisted in using old polychrome-

blue prepared after Unna's formula, commercial polychrome-blue of Unna, old polychrome-blue prepared after Goldhorn's formula, freshly prepared polychrome-blue after Unna's formula, commercial "Goldhorn's polychrome methylene-blue," solutions of medicinal methylene blue, solutions of polychrome-blue in which the methylene blue and sodium carbonate varied from 0.1 % to 1 %, solution prepared according to Nocht's method No. 3, with silver oxide (similar to Borrel's blue), and in changing the order of mixing. A polychrome-blue solution containing only 0.5 % of methylene blue and 0.5 % sodium carbonate may be used, but deeper chromatin staining is obtained with mixtures containing from 0.75 % to 1 % of each.

It was found impossible to obtain a polychrome-blue solution from simple methylene blue (Grübler) and sodium carbonate, a dirty brown precipitate being thrown down on heating.

Samples of eosin other than the water-soluble yellow eosin (Grübler) did not give good results.

Finally, from the above, the following directions for preparing a satisfactory stain were deduced. Three solutions, a 1% aqueous solution of water-soluble yellow eosin (Grübler), freshly prepared polychrome methylene-blue solution, and 1% aqueous solution of Ehrlich's rectified methylene blue (Grübler) are mixed together. The freshly prepared, partially neutralized polychrome-blue never fails to give good results. An unneutralized polychrome-blue solution may be used. It is unnecessary to cool the freshly prepared polychrome-blue solutions. The solutions should be mixed together in the following order:

A. Distilled water . . . . . 1000 c. c.
B. 1% aqueous sol. of eosin (water soluble, yellow) . 100 c. c.
C. Polychrome-blue sol. (freshly prepared) . . . 200 c. c.
D. 1% aqueous sol. of methylene blue (Ehrlich's rectified). 70 c. c.

A greenish metallic scum appears on the surface and a fine black precipitate is thrown down. This precipitate may not appear until 80 c. c. of solution D have been added.

The mixture is filtered at once or after standing for from twenty to thirty minutes and the residue allowed to dry in the air (from twenty-four to forty-eight hours), or is dried in a hot air chamber at a temperature not above 60° C. The dried residue is scraped from the filter paper, powdered in a mortar, and dissolved in pure methylic alcohol.

From the above amounts of unmixed solutions about 0.7 to

1 grm. of powder will be obtained, of which 0.25 to 0.3 grm. will saturate 100 c. c. of pure methylic alcohol (Merck's). To obtain complete solution of 0.25 grm. of powder in 100 c. c. of methylic alcohol, it is necessary to mix them in a mortar, using considerable pressure to break up the powder; intense colors varying from blue to a reddish-purple upon the sides of the mortar and the pestle will show that proper solution has been obtained.

Some lots of methylic alcohol show an acidity of 1 to 2 c. c. $\frac{N}{10}$ alkali to 100 c. c. of alcohol, and such an alcohol should be neutralized with 0.05 to 0.1 grm. of dry sodium carbonate before mixing with the powdered precipitate, since this amount of acid is sufficient to give results similar to over-neutralization.

Over-neutralization destroys the property of staining the chromatin granules, the specimen staining intensely red throughout; and a marked alkaline reaction—i.e., with no neutralization—prevents proper fixation, the red cells and leucocytes appearing blurred and frayed out at the edges, and gives intense blue staining throughout.

When the solution is of proper strength it has a purple-plum color, and after shaking leaves the sides of the bottle quite clear above the surface of the stain.

FIXING AND STAINING.

As with Jenner's stain, previous fixation is unnecessary. Blood films on cover-slips or smears on slides are thoroughly dried in the air. The specimen is flooded with the stain in full strength for one minute, then diluted with a few drops of distilled water (five or six drops for a $\frac{7}{8}$ in. cover-slip) until the greenish metallic scum appears on the surface, and one can readily see through the diluted solution at the edges of the cover-glass only when the specimen is held over a white surface (filter paper). The diluted stain is allowed to remain on the specimen for five minutes (Leishman) in order to bring out the eosin-staining of the red cells, the granular staining, and the polychrome staining properties; the preparation is then washed in distilled water for two or three seconds and dried immediately by blotting.

For malarial specimens this procedure is always sufficient to stain the young forms, but the mature forms of tertian and quartan and the crescents of æstivo-autumnal fever may require two minutes' flooding with the undiluted stain and ten minutes' flooding with the diluted stain for the bringing out of the chromatin particles in the parasites.

The negative surface of the specimen should be carefully inspected and washed, if necessary, since the stain drying on the glass gives rise to a thick greenish coating which obscures the field of examination.

This method does not employ the washing out of the blue spoken of by Wright, but does include his "differentiation," the principle of which we first learned from Leishman's publication. If the specimens are washed and dried after staining one minute in full strength, the staining of the nuclear material is of a pale blue, and of the red cells and of the granules of the leucocytes faint or none.

The dilution of the stain accomplishes the "differentiation" desired, unless the dried specimens have been kept for several weeks or months; these old specimens always stain a deep blue throughout.

The normal red cells in well-spread specimens vary in color from a dull light red to a deeper eosin red. If after staining for one minute in full strength the water and alcoholic stain be not thoroughly mixed in diluting, particularly in thickly spread areas of the specimen, the red cells may show a bluish tinge or absence of the red stain in streaks.

Polychromatophilia and granular basophilia are well shown. The "stippling" (Tüpfelung) (Plate XXI, Fig. 31) of Schüffner (21) and Ruge (22) and Goldhorn (23), appearing in many red cells invaded with tertian parasites, is of a color decidedly different from that of the basic granules (Plate XXI, Fig. 26) of the anæmias and lead-poisoning; and the occurrence of both these changes in the red cells, but never together in the same red cell, in cases of malarial infection with well-marked secondary anæmia, differentiates them. The "stippling" varies somewhat with the reaction of the staining solution, increasing in intensity and in

extent (i.e., extending to cells containing young as well as cells containing old parasites) with the increase in alkalinity of the stain. The unneutralized stains always bring out marked "stippling," the cells containing the parasites showing an irregular spiculated edge somewhat similar to certain crenated corpuscles, a fine cytoplasmic reticulum, and small nodal dots stained a deep crimson-red. The non-invaded cells, however, do not show "stippling" even with a stain sufficiently alkaline to interfere with fixation and staining. "Stippling" is evidently a specific cell-degeneration.

The occurrence of azurophile granules in the protoplasm of many of the mononuclear forms (small and large) is found in all specimens.

The staining of eosinophile granules is not so clear and bright as with other stains (eosin and methylene blue, or Jenner's) and may prove puzzling to one not familiar with the blood. The variety of colors cannot well be described, and an idea of them is best obtained from specimens, and from the drawings attached.

NUCLEAR STAINING.

The nuclear material of white cells and of nucleated red cells takes a dark purple-plum color, that of the erythroblasts tending more toward dark-blue tints. More characteristic than the tint is the clearly defined staining of the chromatin material, resulting in nuclear pictures excelled in clearness by good hematoxylin staining only. The chromatin-thread staining is not well represented in the colored figures, but is easily seen in the photographs of megaloblasts (Plate XXII, Figs. 1–4) and of the mitotic myelocyte (Plate XXII, Fig. 5). Trachychromatic and amblychromatic nuclei are not well differentiated, and to us this does not seem a characteristic necessary to a good stain, since it is a point of differentiation allowing of extreme variations due to personal equation, and is, therefore, one of small value in classifying cells. The clearness or unclearness of staining is, however, important, and Nocht's dyes bring out definitely clear-cut, deep-staining nuclei in the majority of

leucocytes, and poorly stained nuclei not sharply outlined in a small number of cells—the former evidently healthy nuclear material, the latter examples of hydrops of the nucleus. Unless one bears this in mind and at the same time rids one's self of the importance of pale- and deep-staining nuclei in differentiating types of cells, the mononuclear forms will often be confusing and more rarely polymorphonuclear and mononuclear forms will be confused.

Definitely amblychromatic nuclei occur in mast-cells and myelocytes, and yet here the shape of nuclei, relation of nucleus to cell-body, and granulations are the important factors in deciding upon the type of cell.

These remarks in regard to nuclear staining are dwelt upon, for during the last two years while using modified Nocht's dyes and studying nuclei carefully we have not found myelocytes in the peripheral circulation nearly so frequently as with the older dyes (eosin and methylene blue, Ehrlich's triple stain, Jenner's stain).

The granular leucocytes, using the qualification granular in its original sense, exhibit three characteristic staining reactions, viz., of nucleus, of ground-substance of protoplasm, and of the granules. The ground-substance of the neutrophilic and eosinophilic cells takes a decided pink stain, and the granules decided red tints, that of the eosinophile being more red than that of the neutrophile. The mast-cells show an unstained, white ground-substance in which are scattered the densely stained purple granules. The transition forms (Plate XXI, Figs. 16 and 16a) show a blue-staining ground-substance, deeper in tint than the blue of the large mononuclear forms and lighter than the blue of the lymphocyte protoplasm, with fine granules scattered over the blue background, these granules in some cells suggesting the "azur-granulation" and in other cells the neutrophilic granulation.

BASIC STAINING.

Nuclear staining has been briefly considered separately, for in the azur-stains the nuclear tints are probably due either to the metachromatic property of the stain or to a combination of

methylen-azur and eosin staining, the eosin succeeding the methylen-azur in its reaction with the nuclear material, while true basic staining is found in the protoplasm of the lymphocytes and in the mast-cell granules. In normal blood the lymphocyte (Plate XXI, Figs. 5–9) alone shows the deep blue protoplasm characteristic of the reaction of a simple basic blue dye; the mast-cell granules (Plate XXI, Figs. 22 and 22a) are metachromatic as well as definitely basic, varying in tint from a deep blue to a decided purple or even a deep red tint. The protoplasm of the large mononuclear cells reacts faintly basic, the tint varying from the faintest blue to a decided light blue.

In pathologic blood, in addition to the normal lymphocyte, two types of cells with deeply staining basic cyanophilic protoplasm are found, the "stimulation-form" of Türk (Plate XXI, Figs. 13 and 13a) and the "large lymphocyte" or lymphoid-marrow cell of acute "lymphatic" leukæmia (Plate XXI, Fig. 21). All myelocytes (Plate XXI, Figs 18–20) show a definitely basic blue ground-substance, and a few of them show basic granules similar to the mast-cell granulation.

NEUTRAL STAINING.

The granulation of finely granular polynuclears reacts with a less red tint than that seen in the eosinophiles—a red with a shade of blue, not the violet or lilac tint so characteristic of the other good neutral stains (Ehrlich's, Jenner's), so that the size and shape of the individual granule are more characteristic of this cell than the staining reaction. A similar color reaction is evident in some of the transition forms, the "transitional neutrophiles" of Cabot (Plate XXI, Fig. 16), which contain granules having no affinity for an uncombined "azur" dye, as "azur blau" in methyl alcohol. The granules of the finely granular myelocytes react as do the granules of the polynuclears, while the myelocytic granulation is, as a rule, not so abundant as in the polynuclear cells.

AZUR STAINING.

The most characteristic property of the stain is the staining of certain granules in large mononuclear (Plate XXI, Figs. 10, 11, 14,

15), transitional (Plate XXI, Fig. 16a), and some lymphocyte-like cells (Plate XXI, Fig. 14a), which with simple acid and basic dyes and neutral dyes (as Ehrlich's and Jenner's) show no granulation in the protoplasm. The same reaction is found in certain granules in the protoplasm of the "large lymphocytes" of acute lymphatic leukæmia. Large mononuclear cells, with and without this "azur-granulation," occur, the former being not numerous. Such granulation may be considered due to the fragmentation and dissemination throughout the protoplasm of nuclear particles, or as due to degenerative changes in the protoplasm, the latter view being that of Michaelis and Wolff (24), Türk (25), and Ehrlich (26). This degenerative change occurs in large mononuclear forms and in lymphocytes, so that such a cell as 14a, Plate XXI, is an old or aged lymphocyte which stained in its younger hours as a typical lymphocyte (Plate XXI, Fig. 5).

In many specimens of blood, however, we can readily find cells of the large mononuclear type, the protoplasm of which exhibits a constricting and budding process, resulting in the formation of a cell with a nucleus staining deeply and surrounded by a narrow zone of light-blue protoplasm containing few (from 3 to 6) or many (from 30 to 40) "azur granules." Certainly a large number of such cells are not aged lymphocytes but atypical large mononuclears. Similarly one finds not infrequently constricting portions of transition-forms (Plate XXI, Fig. 16a) simulating plates (Plate XXI, Fig. 2b) in size, but not typical in staining reaction, the resemblance being sufficiently close, however, to be confusing. These false-plates seem to us to correspond to the "large" plates described by some observers.

STAINING OF PLATES.

Excepting the plate-like bodies derived from large mononuclear and transition forms, the plates (Plate XXI, Fig. 2a), particularly in specimens prepared with two per cent. solution of sodium metaphosphate, present a faintly pink outer ring or

zone surrounding a faintly blue inner portion in which there is a deep red rod-like reticulum. In most specimens the plates in masses present only the deep red reticular structure, but in these same specimens a few scattered individual bodies will show the characteristic staining reaction of the plates in blood prepared with sodium metaphosphate.

CHROMATIN STAINING.

The chromatin material of the malarial parasite (Plate XXI, Figs. 27–30) takes the deep ruby-red tint so characteristic of the original Romanowski stain. With Nocht's methods, however, the chromatin staining is much more certain than with the modifications of Romanowski's method (Wright's, Leishman's).

In the younger phases of growth the chromatin stains deeply and clearly, while in the older, full-grown, crescentic and ovoid forms (i.e., in the gametocytes) the chromatin rods stain faintly red. The chromatin material in the nuclei of amœba coli, of trypanosomes, and of the Leishman-Donovan body resembles that of the malarial parasite in staining reaction.

CLASSIFICATION OF WHITE CELLS.

Study of blood, marrow, and lymphoid-tissue specimens with the older and then the "azur" dyes impresses one with the correctness of the views of Ehrlich and Türk as opposed to the classification elaborated, or simplified, if one so chooses, by the school headed by Pappenheim and Grawitz.

Following Ehrlich and Türk, the "azur"-granular lymphocyte-like cell (Plate XXI, Fig. 14a) is to be classed with the lymphocytes, as an aged lymphocyte, although Michaelis and Wolff were not able to find this cell in lymph nodes. Many of these cells undoubtedly are from the larger mononuclear forms, as mentioned under "azur staining"; and partly for this reason, and partly because variations of these cells in the peripheral circulation in

disease may throw some light on their proper classification, we have during the last three years included these "azur" cells with large mononuclear forms in differential counting. In the early stages of variola, before the pustular eruption, this is the type of large mononuclear cells relatively and absolutely markedly increased in number; and likewise in chronic malarial infection, between paroxysms, and in the late weeks of typhoid fever it is increased.

The transition forms characterized with the older stains by the indented or lobed nucleus and by non-granular protoplasm are evidently of two types (Plate XXI, Figs. 16, 16a, 17), the non-granular (Fig. 17), and the granular, some of the latter having pale protoplasm and a definite "azur" granulation (Fig. 16a); others having a more deeply stained protoplasm with a fine apparently neutrophilic granulation ("transitional neutrophiles" of Cabot, "neutrophiles with lobed nuclei" of Türk) are similar to transition phases from the myelocyte to the polynuclear cell found in marrow. This last neutrophilic transition form one finds increased in the peripheral circulation in subsiding infections which produce a polynucleosis and in myelocytic leukæmia. The "azur granule" transitional cell, the supposed end stage of the large mononuclear, is rarely found increased in the peripheral circulation.

The "large lymphocyte" with azur granules (Plate XXI, Fig. 21) of acute lymphatic leukæmia is a cell best classed by itself until we know more of its nature and origin. The presence of definite nucleoli by Nocht's and other methods (methyl-green and pyronin) and the "azur" granules in the protoplasm are characteristic. So far we have encountered such a cell in infant's blood and in acute lymphatic leukæmia. The large-sized lymphocytes of chronic lymphatic leukæmia and of lymphocytosis contain no azur granules. This cell corresponds to Türk's lymphoid-marrow cell, although Türk states he has never found "azur granules" in the protoplasm.

Unfortunately in the three cases of acute lymphatic leukæmia from which we have studied the blood post-mortem examination was not permitted.

The white cells represented in Plate XXI are probably best classified as follows:

In normal blood:	
Polynuclear neutrophiles	Figs. 1, 2.
" eosinophiles	Figs. 3, 4.
Lymphocytes	Figs. 5–9.
Small	Figs. 5–7.
Large	Figs. 8–9.
Large mononuclears	Figs. 10, 12, 14, 14a, 15.
Transitionals	Figs. 16, 16a, 17.
Mast-cells	Figs. 22, 22a.
In pathologic blood:	
Myelocytes	Figs. 18–20.
Neutrophilic	Fig. 18.
Basophilic	Fig. 19.
Eosinophilic	Fig. 20.
Lymphoid-marrow cell	Fig. 21.
Stimulation forms	Figs. 13, 13a.

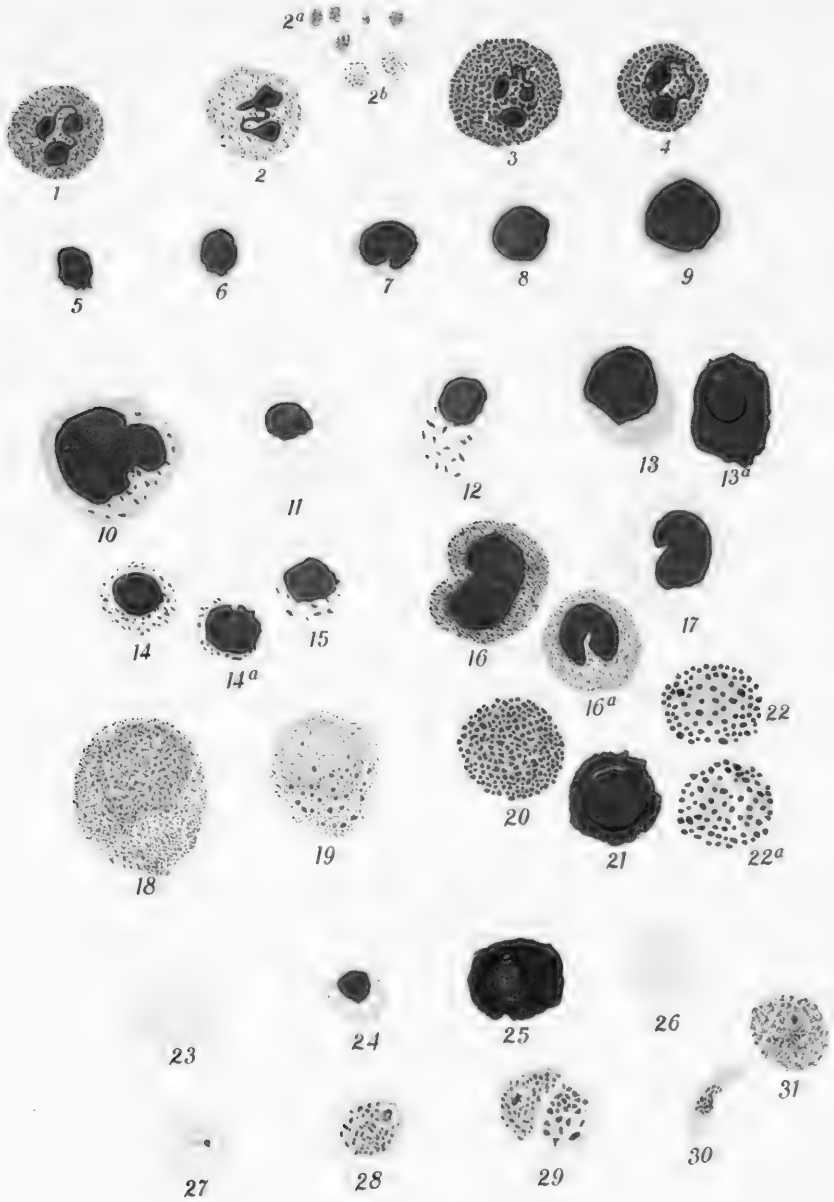
EXPLANATION OF PLATES.

PLATE XXI.

Figs. 1–2.	Polynuclear neutrophiles.
Fig. 2a.	Plates, Fig. 2b False-plates.
Figs. 3–4.	Polynuclear eosinophiles.
Figs. 5–9.	Lymphocytes.
Figs. 10–12, 14, 14a, 15.	Large mononuclears.
Fig. 14a, "old, aged"	lymphocyte of Michaelis and Wolff.
Figs. 13, 13a.	Stimulation form of Türk.
Figs. 16, 16a, 17.	Transition-forms.
Figs. 18–20.	Myelocytes.
Fig. 21.	"Large lymphocyte" of acute lymphatic leukæmia (Türk's lymphoid-marrow cell).
Figs. 22, 22a.	Mast-cells.
Fig. 23.	Normal red cell.
Fig. 24.	Normoblast with basic granules in protoplasm.
Fig. 25.	Megaloblast.
Fig. 26.	Granular basophilia in red cell.
Figs. 27–30.	Malaria parasites.
Fig. 31.	Stippling in red cell invaded by tertian parasite.

PLATE XXII.

Figs. 1–2.	Megaloblasts showing vesicular nuclei (× 1000).
Figs. 3–4.	Mitotic figures in megaloblasts (× 1000).
Fig. 5.	Mitotic figure in neutrophilic myelocyte (× 1000).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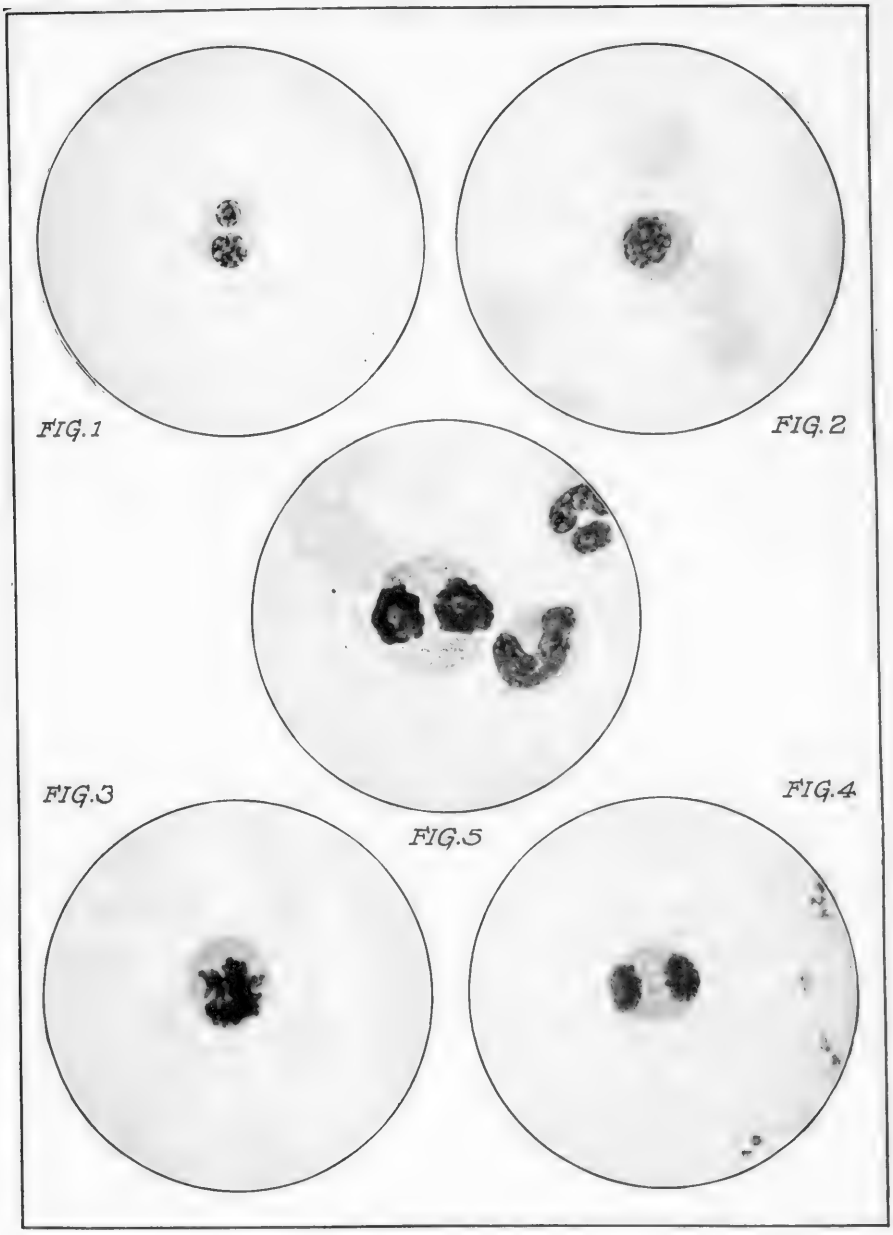

FIG. 1

FIG. 2

FIG. 3

FIG. 4

FIG. 5

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STUDIES ON THE TOXICITY OF BILE.

I.

THE EFFECTS OF INTRAVENOUS INJECTIONS OF BILE UPON BLOOD PRESSURE.

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(*From the Rockefeller Institute for Medical Research, New York.*)

Plates XXIII–XXVI

In a recent communication by Arthur Edmunds,[1] it is stated that the fall of blood pressure produced by intravenous injection of bile salts is insignificant. The statement is supported by the reproduction of tracings obtained from such experiments. The one obtained after an injection of 5 cubic centimeters of a 1 per cent. solution of sodium glycocholate shows hardly any depression. The depression seen on the tracing obtained from an injection of 5 cubic centimeters of a 1 per cent. solution of sodium taurocholate corresponds to a fall of not more than 15 or 20 millimeters of mercury. The author states further that the subject was investigated in the same laboratory [2] a few years before by Dr. A. Young, who also found that the effect of bile salts on blood pressure was very slight. With 5 per cent. solutions of the salts the fall observed by him was hardly greater than that shown in the figures presented by the present author.

There have not been very many studies of the effect of bile upon the blood pressure, and of those made only few workers have given particulars or reproduced kymographic tracings. L. Traube,[3] who was the first one to study the blood-pressure effect of these salts by means of the kymograph, states that an intravenous

[1] Arthur Edmunds, *Brit. Med. Journal*, 1905, i, 57.

[2] Physiological Laboratory of King's College, London.

[3] L. Traube, *Berl. klin. Wochenschrift*, 1864, i, 85.

injection of 4 to 6 cubic centimeters of a 33 per cent. solution of "Natrium choleinicum" produces a considerable lowering of the blood pressure, but he fails to give figures or to reproduce tracings presenting the character and the degree of the reduction of the pressure. M. Loewit,[4] who injected sodium cholate intravenously, states that the effect upon blood pressure is variable, depending upon the dose injected as well as upon the individuality of the animal. Besides Edmunds, Loewit is, as far as we know, the only one who published tracings presenting the lowering of the blood pressure, and the strongest effects which the latter author obtained were apparently not greater than those which were obtained by Edmunds. There are a few other writers who have reported the lowering effect of bile salts upon the blood pressure (Landois, Röhrig, Feltz and Ritter, and others), but their statements are not precise enough to permit a comparison of the degrees of the depressing effect. We have to add that nearly all the authors who studied the blood pressure confined their investigations exclusively to the effects of bile salts. There is nowhere any definite statement as to the effect of pure bile upon the blood pressure.

The results to be presented here were derived nearly exclusively from experiments made by intravenous injections of filtered ox bile or solutions of inspissated ox bile. We have chosen the whole bile in the first place for economic reasons, as taurocholic and glycocholic acids are too expensive for use in considerable quantities in a large number of experiments. But we wish also to record the statement that we have reason to doubt the validity of the now nearly universal belief that all the physiological and toxic effects of bile are due to the bile salts alone. It may be considered as proven that neither the bile pigments nor cholesterin nor any other constituent of the bile is capable of producing effects similar to those produced by the bile salts. But it is far from being proven for bile, as in fact it is not proven for any biological product, that the effect of the sum of all hitherto known parts is equal to the effect of the whole. Our doubts as to the absolute identification of the effects of bile with the bile salts

[4] M. Loewit, *Zeitschrift für Heilkunde*, 1881, ii, 459.

arose in the course of another series of experiments on the toxicity of bile, and we shall therefore not enter here upon a discussion of the real merits of these doubts.

Our experiments were made on rabbits which were anæsthetized by ether. The injections of bile were made from a burette into the jugular vein and the blood pressure was taken from the carotid artery and transmitted by means of a mercury manometer to a rotating drum. The tubes connecting the artery with the manometer were filled with a solution of carbonate of soda or with a mixture of carbonate and bicarbonate of soda.[5] In some experiments tracheotomy was also performed so as to be in readiness for artificial respiration.

We may state at the outset that our experiments brought out often enough both extremes of action. Sometimes the injections of a good quantity of bile would exert only an "insignificant" influence upon the blood pressure, while at other times even a comparatively small dose of bile would produce a "considerable" fall of the pressure, and this occurred on the same animal and with the same concentration of the bile solution. However, these variations in the effects could be produced at will, for they depended largely upon the rate with which the bile was introduced into the circulation. We can therefore subscribe to the statement of Loewit that the dose of the injected bile exerts a distinct influence upon the degree of the effect, and admit also that the individuality of the animal may have something to do with the results. It might be added further that the concentration of the bile is a very important factor, and the general condition of the animal and the original height of the blood pressure, etc., are also factors in the reaction of the blood pressure to the introduction of bile. But all these factors together cannot compare with the importance of the rate of injection on the effect of the bile upon the blood pressure. Ten cubic centimeters and more of concentrated bile can be introduced into the veins of a rabbit without reducing the blood pressure by as much as 15 or 20 millimeters of mercury

[5] Under no circumstances should magnesium sulphate be used in the experiments. The reasons for this caution will be discussed in a future paper.

if only the injection be carried out slowly. On the other hand, even 2 or 3 cubic centimeters of a moderately concentrated solution of bile are capable of reducing the blood pressure by 60 or 70 millimeters when it is permitted to flow rapidly into the vein.

Furthermore, the rapid injection of even a small quantity of bile may have a fatal effect upon the rabbit. Thus the question of the rate of injection may serve not only to reconcile the divergent statements with regard to the influence of bile upon blood pressure, but it may also clear up the contradictions which abound in the literature regarding the fatal effects of an intravenous injection, at least so far as the immediate effects are concerned.

In the early part of the last century it was stated by Magendie and other investigators that an intravenous injection of bile is invariably fatal to the animal. However, the bile which was used was not filtered and it was later assumed that the death of the animal was due to emboli produced by the impurities of the bile. Bouisson reported that rabbits which received 6 cubic centimeters of filtered bile survived the injections. During the last fifty years numerous experiments were carried out with clear solutions of biliary salts, free from corpuscular and from muciginous elements. In the literature on this aspect of the subject we nevertheless meet the same puzzling conflicting experiences. Some authors, such as Frerichs, for instance, have never seen any fatal results from the injection of bile except when air entered the veins. Others, like Leyden, Von Dusch, etc., lost at times some of their animals immediately after an injection of only 1 cubic centimeter of a 10 per cent. solution of bile salts. Von Dusch lost some of his rabbits two or three minutes after injecting a few cubic centimeters of filtered ox bile.

Judging from our experience we have reason to believe that all these contradictions have their chief origin in the variation of the rate with which the injections were made. Six to seven cubic centimeters of bile seem to have been the largest quantity which was ever injected into a rabbit. We have injected into

one rabbit as much as 20 cubic centimeters of a 50 per cent. solution of ox bile within forty-four minutes, and not only was the rabbit apparently in a normal state at the conclusion of the injection but the circulation was even improved; the blood pressure having risen from 100 millimeters of mercury at the beginning to 118 millimeters at the end of the experiment. By extending the injection over a longer period, we have in another experiment injected 50 cubic centimeters of a 50 per cent. solution of ox bile without any visible immediate detriment to the animal. On the other hand, a small dose of 2 or 3 cubic centimeters of bile sufficed to kill a rabbit almost instantaneously when injected very rapidly. We have made, intentionally and unintentionally, a number of such experiments. The blood pressure would sink rapidly to so low a level that even immediately instituted artificial respiration failed to restore it. (Fig. 1 and Fig. 5, Plate XXIII.) The animals died frequently while under respiratory convulsions, which explains the statement of some writers that intravenous injections of bile often cause immediate convulsions.

That the rapidity with which the injections are carried out is an important factor in the results obtained is of course not a new idea. It has often been mentioned by writers dealing with the effects of injections of active substances, and was especially mentioned in connection with the recent studies on the toxicity of urine. But this factor has not been mentioned, at least as far as we know, by writers dealing with the effects of injection of bile upon the circulation. We missed especially allusion to it in the article of Edmunds, since in dealing there with the effects of potassium chloride the tracing given shows that the quantity of the salt which when injected within eight seconds caused only a moderate depression, caused when injected within two seconds a fatal fall of the blood pressure.

That the considerable fall of blood pressure which is caused by a rapid injection of bile is due to the toxicity of the bile and not to the mechanical effect of the injection we have proved by rapidly injecting a solution of 0.9 per cent. sodium chloride. An injection of 10 cubic centimeters or more of sodium chloride

in 20 or 30 seconds marks hardly any change on the blood-pressure curve, while less than half this amount of bile injected within a similarly short time is surely fatal to the animal. In Fig. 2, Plate XXIII, at 1, 10 cubic centimeters of sodium chloride were injected in 10 seconds, and at 2, the same amount of the normal salt solution was injected within 20 seconds. In both cases there was scarcely any effect. Eight minutes later, 4.5 cubic centimeters of pure bile (at 3) were injected within 30 seconds, and the blood pressure sank rapidly, the animal dying within 3 minutes after the injection.

The same experiment also serves to demonstrate that the fall in blood pressure is not due to the low temperature of the bile, since the salt solution as well as the bile was injected at room temperature.

An analysis of our results brings out instructive details. The suddenness with which the blood pressure falls and the steepness of the descending branch in the blood-pressure curve depend essentially upon the rapidity of the injection. In rapid injections the fall of pressure sets in immediately at the beginning of the injection, and only exceptionally is it preceded by a very short slight rise (Fig. 2 at 3, Plate XXIII). The curve representing the fall is the steeper the more rapid the injection. If the injection was made more slowly either at the beginning or at the end, or at any phase, this fact appears well marked on the curve; and when after a considerable fall the injection was slowed, the pressure began to rise before the injection was finished (Fig. 4, Plate XXIII). In rapid non-fatal injections the blood pressure begins to rise immediately on cessation of the injection, and the ascending branch of the curve becomes for the most part a counterpart of the descending branch. In other words, the blood pressure rises at the same rate as it fell. Sometimes the pressure rises a little above the original level only to fall again immediately, for a short period, below this level. A momentary rapid rise to a point above the original height may occur even in cases of a fatal drop of blood pressure (Fig. 5, Plate XXIII).

If care be taken to avoid considerable depressions, rapid injections may often be repeated and kept up for a comparatively

long period without affecting the mean level of the pressure; indeed in some experiments the blood pressure was at the end higher than at the beginning.

When the injection is carried on slowly there may at first be no effect at all. Later the respiratory, Traube-Herring, and other waves of the normal blood-pressure curve disappear one after another and the blood pressure commences to descend slowly. When a slow injection is extended over many minutes, the blood pressure may become reduced by 50 or more per cent. However, the curve representing the fall is not throughout a straight line; the more it descends the more it becomes concave at its upper aspect, which shows that the depressing effect of the bile is gradually diminishing (Fig. 3, Plate XXIV). Nevertheless, even with slow injections, if continued too long, a fatal level from which recovery is no longer possible may be reached. When a slow injection is interrupted, the beginning of the return of the pressure is sometimes delayed a little; but when the return begins the ascending branch of the curve here too is nearly a counterpart of the descending branch; it rises at first slowly and then more rapidly, and the last result, as a rule, is the return of the waves. In slow injections, however, the return is not entirely complete, and the oftener the injection is repeated the lower the level of mean pressure becomes, so that at the end of such an experiment, even if not very prolonged, the blood pressure may be much lower than at the beginning. However, this result is met with chiefly in those experiments in which the injection extends over longer periods of time.

While the rapidity of the fall thus depends essentially upon the rapidity of the injection, the degree of the fall is greatly influenced by the concentration of the bile. For instance, 1 cubic centimeter of a 2 per cent. solution of ox bile injected within ten seconds will reduce the pressure only by 10 to 12 millimeters (see Fig. 7, Plate XXIV), whereas 1 cubic centimeter of undiluted bile injected at the same rate will reduce the pressure by 30 millimeters and more (Fig. 3, Plate XXIV), and 1 cubic centimeter of a 20 per cent. solution of inspissated ox bile injected within 60 seconds will reduce the pressure by 50 or 60 milli-

meters (Fig. 8, Plate XXIV), and the same solution injected in 20 seconds will surely be fatal to the animal (Figs. 1, 2, and 9, Plates XXIII and XXV).

These facts again show that the fall of blood pressure is due to the specific effects of the bile and not to mechanical causes or to the lower temperature of the injected fluids.

From a few observations we have made it would seem that the general condition of the animal and the original height of the blood pressure are factors also influencing the degree of fall produced by the injection of bile. Furthermore, in some cases the injection of bile caused immediately a slowing of the heart-beat, and in those cases the blood pressure reacted more readily and sometimes fell rapidly to a fatal level. In them the descending curve is very steep and shows only a few heart-beats (Figs. 1 and 5, Plate XXIII).

All writers agree that it is the detrimental effect of the bile upon the heart which causes the fall in the blood pressure, although there is disagreement as to whether the heart muscle, or the heart ganglia, or both, become affected by the bile. As regards the nature of the affection, Traube, Leyden, and others believe it to be malnutrition of the heart caused by the destructive action of the bile upon the blood corpuscles. There is no longer any doubt that bile is a strongly hæmolytic substance, but it is an open question whether this factor is especially active in causing the drop in blood pressure. Even at body temperature it takes some time for bile to produce perceptible hæmolysis, whereas the injection of bile into the circulation may produce a fatal drop of blood pressure in a few minutes. We have, however, disproven the hæmolytic theory by a direct experiment. By injecting rapidly a few cubic centimetres of bile, the blood pressure dropped quickly to a fatal level (Fig. 1, Plate XXIII) and the animal died in less than two minutes. The blood was immediately taken from the right ventricle and mixed with normal salt solution; no trace of hæmolysis could be discovered. It can be safely assumed, we believe, that hæmolysis has no share in the fall of blood pressure and the sudden death of the animal following immediately upon the injection of bile.

The autopsies performed immediately after such sudden death have not shown definitely the cause of death. In one case there was considerable pulmonary œdema, the lungs were very hyperæmic, and the right ventricle contained a clot. In most of the autopsies, however, the findings were negative except that both ventricles were distinctly dilated and flabby. The heart's blood was either fluid or was firmly clotted in both ventricles. The lungs were normal. The appearance of the heart was suggestive of paralysis; and if this actually existed it may be conceived either as being due to anatomical changes in the organ or as being the expression of a functional effect, such as inhibition upon the heart's action. As regards the anatomical changes, it has been shown through various investigations that bile alters the structure of muscle tissue as well as nerve fibres and nerve cells. It would, however, be surprising should anatomical changes, as in the fatal cases on the one hand, take place within a few seconds from the beginning of the injection, and as in the case of non-fatal effects, such a rapid structural restitution on the other hand, occur.

Regarding the inhibitory effect of the bile upon the heart, we wish to report some interesting experiments. Before introducing the bile in certain rabbits, one vagus (left) was cut and the minimum strength of electrical stimulus, which caused a distinct inhibitory effect by application to the peripheral end, was established. For the induction coil we used one Daniell cell, and its distance was 300 and 250 millimeters in the rabbits in which this experiment was carried on. A dose of bile, which caused a considerable fall of the blood pressure, was injected, and during the persistence of the fall the irritability of the peripheral end of the vagus was tested, and it was found that not only had the irritability not suffered but that the effect was stronger than before the injection; moreover, in two instances a stimulus which was ineffective before the injection became effective during the fall of the blood pressure (Figs. 8, 9, 11, and especially Fig. 10, Plates XXIV, XXV, and XXVI). We are not aware that such experiments were made before.

In still other experiments we have made the following in-

structive observations. Bile was introduced in such a manner as to cause a fatal fall of blood pressure and the peripheral end of the vagus was then stimulated. It was established that the inhibitory effect of the vagus remained intact as long as there were any signs of a heart-beat. Even when the pressure was not more than four millimeters and the heart-beat hardly perceptible, stimulation of the vagus caused a standstill of the heart (see especially Fig. 9, Plate XXV). O. Sokolow [6] and Loewit [7] state that vagus inhibition passes off before the heart stops. They have published no tracings and have given no data. Our tracings show that not only does the vagus inhibition last as long as the heart-beat, but that it retains its full irritability to the very end.

Our experiments further demonstrate that while the heart's action rapidly fails under the influence of bile, the inhibitory mechanism so far from being impaired by it may even be improved. This fact would favor the view that the effect of the bile upon the heart is a functional one, or, in other words, that bile stops the heart by inhibiting its activity. We do not mean that bile effects the inhibition through the vagus, because we know that the heart is stopped by bile even after eliminating the inhibitory effect of the vagus by a previous atropinization of the animal. Bile may, however, affect the heart in the same manner as potassium salts, the substances which contain three methyl ammonium groups, (Luchsinger and his pupils) and lactic acid (Gaskell [8]), which "weaken the contractions of the heart and ultimately bring it to a diastolic standstill." We see no reason why this action should not be called inhibition, brought about by some kind of a mechanism which is located in the muscle fibres or in invisible ganglia—whichever one chooses to assume. We may state further that we can see no objection to the assumption that the inhibition of the vagus is carried out through this mechanism and that atropin only interrupts the transmission of the vagus impulses through it. The latter

[6] Sokolow, *Physiol. u. toxicol. Studien am Herzen*, Inaug. Dissert., Bern, 1881.
[7] Loewit, *l. c.*
[8] Gaskell, *Schaefer's Text-Book of Physiology*, ii, p. 222.

assumption would, however, collide with the prevailing orthodox views on the subject upon which we do not wish here to enter into a discussion.

DESCRIPTION OF PLATES.

The tracings were obtained in the usual manner by connecting the carotid artery with a mercury manometer which transmitted the variations of the blood pressure upon a revolving drum covered with smoked paper. The curves read from left to right. Besides the blood-pressure curve, there were recorded on each figure a base line, a time-marking line mostly in four seconds, and sometimes also a line marking the beginning and the end of an injection. For lack of space these lines were omitted in most of the reproduced figures. The bile used was either filtered ox bile, pure or dilute, or dissolved inspissated ox bile.

Fig. 1. 2.5 c. c. of 10 % ox bile rapidly injected into the jugular vein, the injection finished in 4 seconds. The blood pressure immediately dropped deep down and the animal was dead in less than 2 minutes. The blood from the right ventricle showed no hæmolysis.

Fig. 2. At "1" 10 c. c. of 0.9 % sodium chloride were injected in 20 seconds; at "2" the same amount of the same solution was injected within 10 seconds; both injections produced scarcely any perceptible effects upon the blood pressure. At "3" 4.5 c. c. of pure bile were injected in 30 seconds. There was at the beginning a very short slight rise, then a rapid fall of the blood pressure followed. The pulse became slower and artificial respiration was instituted, but the animal died in less than 3 minutes.

Fig. 3. At "1" 1 c. c. of undiluted bile was injected in 30 seconds; there was hardly a perceptible effect upon the pressure; at "2" and "3" each 1 c. c. more was injected in 9 seconds, the falls produced were equal to 25 and 30 millimeters of mercury; a slight rise preceded the fall. At the last point of the fall the injection was finished, and immediately the pressure rose again in the same manner as it fell. At x in "4" the injection of 5 c. c. of bile began and lasted 4 minutes; at x at the bottom of the curve the injection was finished, the low pressure lasting a few seconds, after which the rise began and reached the former level. The end of the curve is not reproduced.

Fig. 4. 2.5 c. c. of undiluted bile injected in 40 seconds. The injection was made at first slowly, then more rapidly, and finally considerably more slowly. The pressure began to rise before the injection was finished. The different rates of injection are well shown on the tracing.

Fig. 5. 2 c. c. of 50 % bile injected in 20 seconds. The pulse became very slow and the pressure fell very rapidly to a dangerous depth. At the end of the injection the pressure rose rapidly to above the original level, only to fall again immediately to a fatal level.

Fig. 6. At the arrow the slow injection of 5 c. c. of undiluted bile began and was finished at the cross at the bottom of the curve in 3 minutes. The

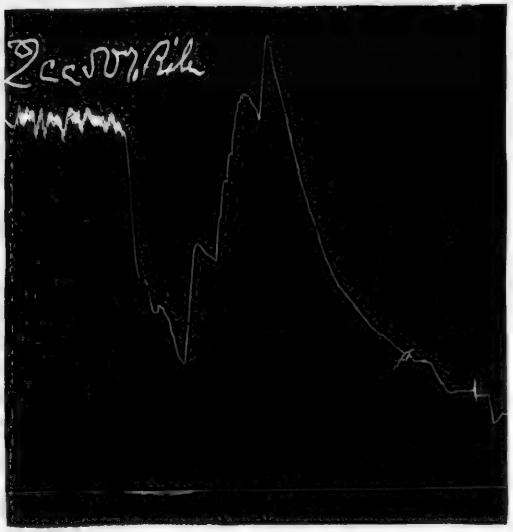


FIG. 5.

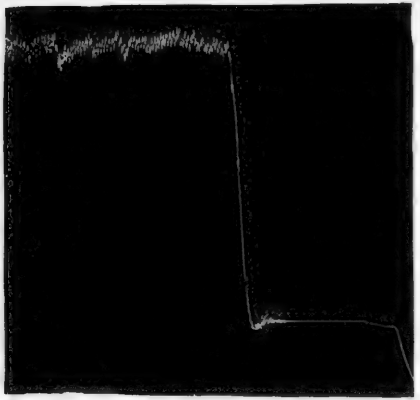
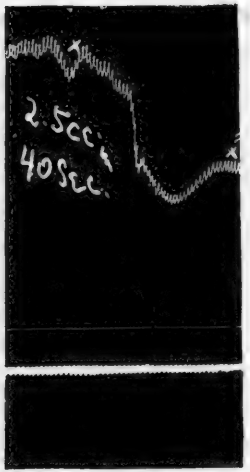


FIG. 1.



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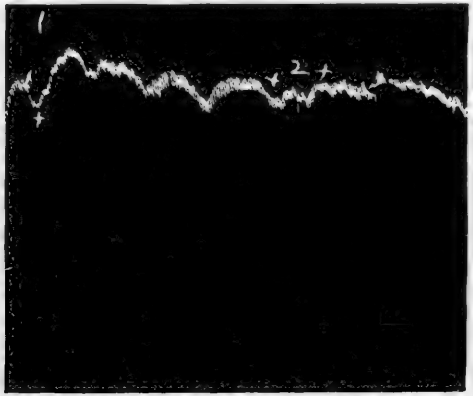


FIG. 2.

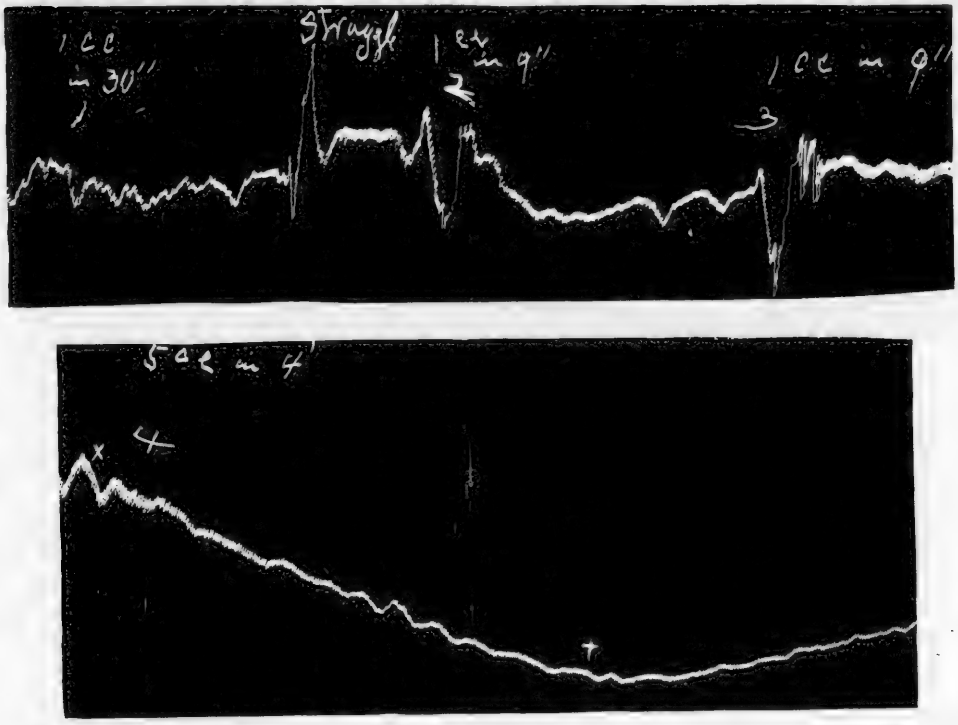
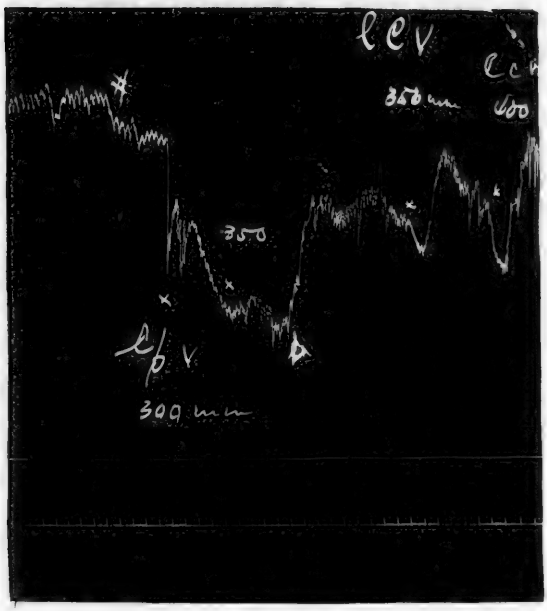


FIG. 3.



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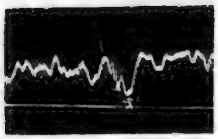

FIG. 7.

Fig. 6.

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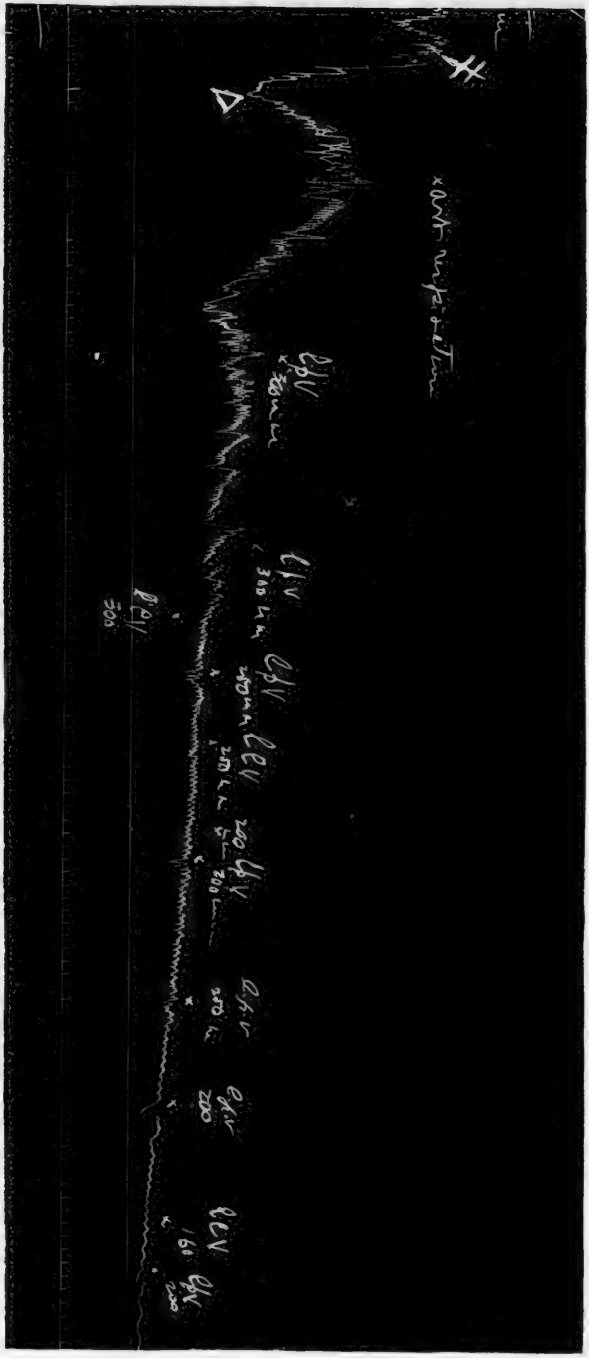

Fig. 9.

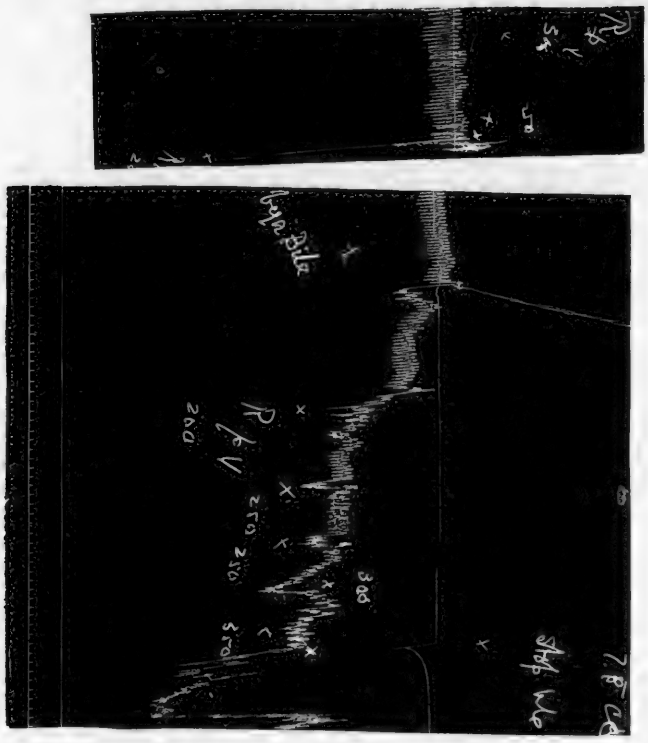

FIG. 10.

FIG. 11.

descent began after the waves disappeared, and the return began a little while after the injection was finished.

Fig. 7. 1 c. c. of a 2 % bile injected in 10 seconds. The fall hardly amounts to 12 millimeters of mercury.

Fig. 8. 1 c. c. of a 20 % solution of inspissated ox bile was injected in 1 minute, beginning at × and finishing at △. At + l. p. v. means that the left peripheral vagus was stimulated by an electric current from an induction when the secondary coil was 300 millimeters distant from the primary coil (Kronecker's induction coil with one Daniell cell). The heart stopped and the blood pressure dropped straight down; 350 mm. distance had no effect. l. c. v. means that the left central vagus was stimulated with 350 and 400 mm. distance at ×, and it caused reflexly a drop which was followed by a rise after discontinuance of stimulation. The middle line is the base line, the lowest line time in 4 seconds.

Fig. 9. 7.5 c. c. undiluted bile injected in 3 minutes. On the left-hand side vagus tested (see description of Fig. 8) before injecting the bile; 300 and 250 mm. distance no effect; 200 mm. causes immediate standstill and a drop of pressure in straight line (number beneath this line). While injecting the bile, 250 and 300 mm. became distinctly effective; a sudden fatal drop followed; artificial respiration ineffective. Line through blood-pressure line marks beginning and end of bile injection. Lower lines, base line, and time-marking in 4 seconds.

Fig. 10. 1 c. c. of a 20 % solution of inspissated bile injected in 20 seconds. × = beginning, △ = end of injection. Blood pressure fell to a fatal level and artificial respiration instituted; blood pressure rose at first, then dropped and continued to sink until 2 mm. above the base line death occurred. During the continued sinking, vagus was tested (see description of Fig. 8). There was still a distinct inhibitory effect when blood-pressure was hardly more than 5 mm., and the heart-beats were barely perceptible. See last mark l. v. p. 200. The two lines beneath blood-pressure curve are base line and time-marking line in 4 seconds.

Fig. 11. 6 c. c. undiluted bile injected in 6 minutes. Fatal fall of blood pressure. Beginning at ×, slight rise preceding the beginning of the fall; △ = end of injection. Vagus tested (see description of Fig. 8); marked inhibitory effect at 300 mm. distance, improved on preceding test which is not reproduced. Lines beneath pressure curve: base line, time-marking line, 4 seconds, slow revolving drum; the line between indicates the time of the injection of the bile.

ON THE HEXON BASES OF LIVER TISSUE UNDER NORMAL AND CERTAIN PATHOLOGICAL CONDITIONS.

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1. Introduction. 2. Method. 3. Hexon bases in normal dog's liver. 4. Hexon bases in the livers of dogs poisoned with phosphorus. 5. Hexon bases in the livers of dogs repeatedly anæsthetized with chloroform. 6. Conclusion.

INTRODUCTION.

The discovery by Drechsel that bases are among the decomposition products of proteid material was an important step for proteid chemistry. Drechsel found the bases lysin—a diaminocaproic acid—and lysatinin, and from the latter Hedin afterwards isolated the base arginin. Later A. Kossel added a third member to the group of hexon bases [1] by the discovery of histidin. The fact that these three bases, lysin, arginin, and histidin, are obtainable from nearly all proteid matter makes their investigation of much interest, and it is a matter of great importance that Kossel and his pupils have been able to devise methods for their satisfactory quantitative estimation.

The interest attaching to a knowledge of the actual yield of the hexon bases relates not merely to strictly physiological questions connected with the proteid molecule. It has long been a question with pathologists whether the various degenerations of parenchymatous cells were not attended by alterations in the constitution of the proteid molecule during life, but the methods heretofore at the disposal of investigators have not permitted any satisfactory answer to be given to the question.

In the hope of throwing some light on these fundamental problems, I have been led, at the suggestion of Professor A. Kossel, to study this condition of the proteid molecule with

[1] So called on account of each molecule containing six atoms of carbon.

regard to the yield of hexon bases in phosphorus poisoning.[2] The results now obtained through the study of the dog's liver in this condition and in chronic chloroform poisoning are sufficiently definite and uniform to justify their presentation, in the belief that they constitute a first step toward the correlation of histological and profound chemical alterations in the proteids of living cells.

The work on the normal and phosphorized dogs was performed in Professor Kossel's laboratory in Heidelberg; the remainder of the work was done in collaboration with Professor Herter, to whom I am indebted for the opportunity of making this contribution.

METHOD.

The final separation and isolation of the hexon bases, in this series of experiments, was carried out according to the method of Kossel and Kutscher.[3]

The principle of the individual separations depends upon the insolubility of the silver compounds of arginin and histidin in a fluid saturated with barium hydrate, lysin remaining in solution, and also upon the insolubility of the silver compound of histidin in a fluid rendered faintly alkaline with barium hydrate, the arginin, under these conditions, remaining in solution.

The early part of the procedure varied somewhat from the method above referred to.[4] The method adopted was as follows:

A nitrogen determination [5] was made upon portions of the fresh organ as well as the dried one, which had been used for water estimation[6]; 150 grams of the finely divided tissue were mixed with 900 c. c. water and 450 grams H_2SO_4,

[2] A preliminary report appeared in the *Berliner klinische Wochenschrift*, 1904, xli, 1065.

[3] *Zeitschr. f. physiol. Chemie*, 1900, xxi, 165.

[4] It may be well to state that an attempt was made to keep the mode of procedure in all the cases studied as much alike as possible, thus obtaining results which, if not absolute, were at least more nearly comparable.

[5] Duplicate nitrogen determinations, according to Kjeldahl, oxidizing by aid of $CuSO_4$ and K_2SO_4, were made throughout the analyses, and a control of the nitrogen content of precipitates and fluids was kept by making nitrogen estimations upon aliquot parts of filtrates, observing correction for fluid taken.

[6] It is interesting to note the slight loss of nitrogen through the process of drying to a constant weight at 105° C. Of fourteen instances of nitrogen determinations upon fresh as well as upon dried tissue of the organ, the average loss of total nitrogen was 0.047 %.

sp. gr. 1.80. The mixture was boiled in a flask with an upright condenser on a paraffin bath fourteen hours. After cooling and diluting, the small amount of fatty matter remaining undissolved was removed by filtration.[7] A hot solution of 1000 grams of crystallized $Ba(OH)_2$ was added to the hot filtrate, thus removing about 70 % of the H_2SO_4. The bulky precipitate was filtered off and thoroughly washed,[8] and the filtrate evaporated to a certain point (approximately one litre), until the H_2SO_4 content was brought to 5 % by volume, this proving to be the most favorable concentration for the subsequent precipitation. At this point, the hexon bases were thrown out of solution as phosphotungstates.[9] The precipitate was filtered off and washed with water containing 5 % by volume of H_2SO_4 and a few drops of phosphotungstic acid. After complete washing, the precipitate was rubbed up with a small amount of water, and decomposed on the water bath with $Ba(OH)_2$ in very slight excess.

The bases, thus thrown back into solution, were removed from the insoluble barium phosphotungstate by filtration and complete washing with hot water, and the slight excess of $Ba(OH)_2$ in the filtrate was thrown out with CO_2.[10] After concentration, the $BaCO_3$ was filtered off and washed, and nitrogen estimated in the filtrate. After acidifying with HNO_3, silver nitrate solution was added in slight excess, as indicated by the yellow-colored precipitate formed when a drop of the clear fluid was brought in contact with a drop of solution of barium hydroxide on a watch glass.

The small quantities of purin bodies which were brought down with the phosphotungstate precipitate separated at this point and were removed by filtration.[11] The clear fluid was then saturated with $Ba(OH)_2$. The precipitate (A) contained arginin and histidin; the fluid (B), lysin.

(A) The precipitate, quickly filtered and washed with baryta water and suspended in weak H_2SO_4 solution, was decomposed with H_2S. After removing the H_2S by evaporation, and the precipitated $BaSO_4$ and Ag_2S by filtration and washing, nitrogen was estimated in a portion of the filtrate. The remainder of the filtrate was neutralized with $Ba(OH)_2$, and $Ba(NO_3)_2$ added to complete the precipitation. The $BaSO_4$ was filtered off and washed. The filtrate was acidified with HNO_3, and $AgNO_3$ added in slight excess. It sometimes occurred that traces of purin bodies separated at this point; if so, they were removed by

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[7] In nine determinations the average percentage of total nitrogen in this residue was 0.049.

[8] The $BaSO_4$ holds back nitrogen in spite of thorough washing. Although in each case the precipitate was washed with over 25 litres of hot water, the average percentage of total nitrogen which was held back was found, as a result of nine determinations, to be 0.111.

[9] In order to insure uniformity, the phosphotungstic acid reagent was always prepared by the method of Drechsel. *Berichte d. deutschen chem. Gesellschaft*, 1887, xx, 1454.

[10] The fluid at this point in every case was of a deep rose color, and this color appeared later, although in less degree with lysin. The nature of the color reaction was not investigated.

[11] Their amount, in terms of nitrogen, varied from 0.02 to 0.037 % of total nitrogen, the average of seven determinations being 0.0273.

filtration. The fluid was carefully neutralized with $Ba(OH)_2$, then more $Ba(OH)_2$ was added cautiously from a burette until a drop of the clear supernatant fluid failed to give any turbidity with a drop or two of a weak odorless solution of ammoniacal silver. The precipitate was quickly filtered and washed. The precipitate (a) contained histidin; the filtrate (b), arginin.

(a) The histidin silver was decomposed in the usual manner with H_2S in the presence of H_2SO_4. The H_2S was removed by evaporation, the Ag_2S filtered off and washed, and a portion of the filtrate subjected to a nitrogen estimation, upon which determinations the values for histidin in the various tables below are based.[12]

The remainder of the filtrate, made to contain $2\frac{1}{2}$ % by volume of H_2SO_4, was treated with $HgSO_4$ in slight excess according to the method of Kossel and Patten.[13] The histidin mercury was decomposed with H_2S, the H_2S driven off by heat, and the HgS removed by filtration. After removing the H_2SO_4 by $Ba(OH)_2$, the excess of $Ba(OH)_2$ by CO_2, and the last traces of $BaCO_3$, the fluid was rendered slightly acid with HCl, evaporated, and allowed to crystallize, the histidin being weighed as the dichloride.[14]

(b) The filtrate containing the arginin was saturated with $Ba(OH)_2$, and the precipitate quickly filtered off and washed with baryta water. After decomposing the silver arginin with H_2S in the presence of H_2SO_4, removing the H_2S and filtering off the precipitated Ag_2S and $BaSO_4$, a nitrogen estimation was made upon a portion of the fluid. The arginin results recorded in the tables below are based upon these nitrogen estimations. From the remainder of the fluid the H_2SO_4 was removed with $Ba(OH)_2$ and the excess of $Ba(OH)_2$ with CO_2. After the last trace of $BaCO_3$ was filtered off, the fluid was neutralized with HNO_3, concentrated, and the crystals of arginin nitrate finally obtained and weighed.[15]

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[12] In view of the report by Kutscher and Steudel (*Zeitschr. f. physiolog. Chemie*, 1903, xxxix, 12) relating to the untrustworthiness of the Kjeldahl method for the estimation of nitrogen in certain definite bodies, among them histidin and lysin, an objection might be raised to this procedure. But of the two choices—basing the results upon the nitrogen estimation, or upon the weight of the final crystallized product—the former is probably the more accurate. I may say that the duplicate nitrogen determinations upon which the histidin and lysin values are based, were, more often than not, in absolute agreement. The results obtained by Sörensen and Pedersen (*Zeitschr. f. physiolog. Chemie*, 1903, xxxix, 513) in their nitrogen determinations of lysin compounds by the Kjeldahl method agree closely with the theoretical requirements.

[13] *Zeitschr. f. physiolog. Chemie*, 1903, xxxviii, 39.

[14] The histidin nitrogen calculated from the weighed histidin dichloride ($C_6H_9N_3C_2$. 2HCl) was, as a rule, somewhat lower than the value based on the Kjeldahl determination. Of nine specimens studied, the average nitrogen was 0.0519 grm. by Kjeldahl, and 0.048 grm. by weight.

[15] The nitrogen based on the Kjeldahl determination and that calculated from the weighed arginin nitrate ($C_6H_{14}N_4O_2$. $HNO_3 + \frac{1}{2}H_2O$) agreed fairly closely. In some instances the latter showed a somewhat higher value, due probably to the unremoved traces of $BaCO_3$. The average of the nine specimens studied gave 0.1772 grm. by Kjeldahl and 0.1850 grm. by weight.

(B) The fluid containing the lysin was acidified with H_2SO_4 and the excess of silver used in the previous precipitation removed with H_2S. After evaporating off the H_2S, the rather bulky precipitate of $BaSO_4$, together with the Ag_2S, was filtered off and washed. After concentrating to a convenient bulk, the lysin was again thrown out of solution with phosphotungstic acid, filtered, washed, decomposed with $Ba(OH)_2$, and the excess of $Ba(OH)_2$ removed by CO_2 in the usual manner, after which a nitrogen determination was made upon a portion of the fluid. The results for lysin recorded in the tables are based upon these nitrogen estimations.[16] From the remainder of the fluid, after evaporating, the lysin was separated as a picrate. After washing, recrystallizing, and weighing the lysin picrate, the lysin left in the mother liquor and washings was again thrown out of solution by phosphotungstic acid. In carrying out this method, the picric acid was first removed with ether, after which the process was repeated as before. The weight of the lysin picrate recovered was added to the weight of the main portion.[17]

Hexon Bases in Normal Dog's Liver.—For the sake of comparison normal values were established by taking the livers of two healthy, well-nourished dogs, weighing 10 and 13 kilos respectively. The dogs were killed, about fourteen hours after feeding, by bleeding. The livers appeared normal. To ascertain if the small amount of blood left in the liver after death by bleeding produced any appreciable effect upon the results, a third normal and fairly well-nourished dog of about 9 kilos weight was taken. After a similar bleeding, the liver of the last dog was washed with five litres of Ringer's solution, thus removing practically all the blood from the tissue. The results obtained are recorded in Table I, Division A.

It is likely that these values are within the range of normal variation. Probably the influence of the small percentage of blood left in the liver tissue after death by bleeding may be disregarded, and, with one exception, in the subsequent examples studied, the last traces of blood were not removed from the liver preparatory to analysis.

Hexon Bases in the Liver of Dogs Poisoned with Phosphorus.—Seeking a condition showing marked degeneration of the liver

[16] See footnote 12, page 295.

[17] The average of the nitrogen values in the specimens studied was 0.1261 grm. by the Kjeldahl method, and 0.1147 grm. by crystallizing and weighing as lysin picrate ($C_6H_{14}N_2O_2 . O_6H_2(NO_2)_3OH$). The amount obtained by the latter method was, as a rule, somewhat less than was indicated on the basis of the Kjeldahl estimations.

cells and one in which we might expect changes influencing the structure of the proteid molecule itself, observations were undertaken upon the liver in phosphorus poisoning.

Two dogs were placed under the phosphorus treatment. The first (No. IV, Table I), weighing 10 kilos, received during a period of 32 days, thirteen subcutaneous injections of a moderately strong solution of phosphorus in olive oil. The first nine injections were of 0.5 c. c. each, and they were followed by one injection of 0.6 c. c., 0.7 c. c., 0.7 c. c., and 0.8 c. c. each. It was not until after the ninth injection, on the twenty-sixth day, that the animal began to show symptoms. One day after the last injection the dog was killed by bleeding. He had lost 1.7 kilos in weight, and the liver showed the appearance of phosphorus poisoning, although in less marked degree than the next animal employed.

This dog (No. V, Table I), weighing 10.3 kilos, received during nine days six subcutaneous injections of 0.5 c. c. each of a saturated solution of phosphorus in olive oil. The third day after the last injection the animal died. A few minutes after death the blood was removed from the liver by washing with Ringer's solution, as in the case of normal dog No. 3. The dog had shown marked symptoms of depression and complete loss of appetite, and diarrhœa; he had lost 1.4 kilos in weight. The liver was extremely fatty, showing the typical appearance of phosphorus poisoning.

The results obtained with these two animals are recorded in Table I, Division B. A comparison of averages may be seen in Table II, and the percentages of diminution of the bases, compared with the normal values, are given in Table III.

In comparing the values obtained from the two phosphorus-poisoned dogs with the values regarded as normal, one is struck with the marked diminution of the hexon bases as a whole in the former. Thus the average nitrogen content of the bases in the normal liver tissue is 17.04 % of the total nitrogen (Table II), while the content of the liver of the phosphorus dog (IV) is only 10.72 %, a falling off of 37.1 % (Table III). In the case of the more advanced phosphorus poisoning (V), the nitrogen of the

hexon bases is only 8.11 % of the total nitrogen (Table II), or a loss of 46.01 % (Table III) from the corresponding normal value of 15.02 % of Exp. III. Of the bases, arginin suffers the greatest relative diminution, and its loss is a trifle more marked in Case V than in the others. Thus the average arginin nitrogen in the liver of the normal dog is 9.62 % of the total nitrogen, while in that of the phosphorus dog (IV) it is only 5.05 % (Table II), a diminution of 47.52 % (Table III). In the washed dogs' livers the normal arginin nitrogen value is 8.72 %, while in the phosphorus dog (V) it is only 4.14 %, thus making a loss of 52.49 %.

It is interesting to note the falling off of the total nitrogen in the cases under consideration. The average total nitrogen in 100 parts of the dry normal tissue is 11.41 (Table II); in the phosphorus dog of Exp. IV, the value is 7.34, a diminution of 35.67 % (Table III). The falling off of the nitrogen of the hexon bases in the same animal is 37.1 % (Table III). This would seem to indicate that that part of the proteid molecule, involving the hexon bases, has not in the pathological tissue undergone a greater relative decomposition than the other nitrogenous matter of the organ. In the case of the dogs whose livers were washed, the total nitrogen in 100 parts of the dry liver tissue fell from 12.48 (III) to 7.9 (V), a loss of 36.7 %. In this instance, however, the proteid matter involving the hexon bases of the phosphorus-poisoned tissue seems to have suffered a slightly greater relative breaking down, amounting to 46.01 % (Table III).

Hexon Bases in Livers of Dogs Repeatedly Anæsthetized with Chloroform.—In the course of some experiments upon dogs which had been repeatedly subjected to chloroform narcosis for longer or shorter periods, the opportunity to study possible changes in the hexon bases of the liver tissue was presented. Such a study seemed not without interest, especially in the light of the facts reported by Taylor to which reference will be made later.

In considering the results of the four instances of chloroform poisoning presented in Division C of Table I, it should be borne in mind that the first two dogs (VI and VII) had been under the

chloroform treatment for a somewhat shorter period than the other two, and showed therefore less advanced histological changes of the liver. Hence the four specimens, roughly speaking, fall into two groups.

The first dog (VI) was a moderately well-nourished animal of 9 kilos weight. It was placed under chloroform one hour daily for five days, after which it was killed by bleeding. Upon histological examination, for which, here as in the subsequent instances, I am indebted to Dr. Wm. R. Williams, the liver showed a moderate degree of fatty degeneration, which was rather more marked toward the centre of the lobules.

The next dog (VII) weighed 7.5 kilos and was well nourished. It was anæsthetized on nine occasions, each lasting about one hour, during a period of fifteen days. The dog ate very sparingly and became much emaciated. At the last administration of chloroform the dog died; but the arteries were quickly severed, allowing most of the blood to escape from the liver. Histological examination showed fairly marked fatty degeneration of the liver cells.

The third dog (VIII), weighing 12 kilos, was subjected to chloroform inhalation three times a week for 58 days, making in all twenty-five occasions of anæsthesia. The first nineteen periods of inhalation lasted one hour each, and the last six, one and a half hours each. Toward the close the animal became much emaciated and suffered a complete loss of appetite. He was finally killed by bleeding.

The histological examination showed marked hyalin degeneration of the liver cells, with considerable formation of new bile ducts, and an abundant production of richly cellular connective tissue.

The last dog (IX), weighing 5 kilos, and only moderately well-nourished, was under the chloroform treatment four weeks, receiving inhalations three times weekly, at first of one hour and later of one and a half hours' duration. The animal rapidly lost weight and became much emaciated. He was found dead in the cage, and hence it was impossible to free his liver from blood. In spite of this drawback, however, it seemed well to examine

the organ for possible changes in the hexon bases. The histological examination revealed very widespread and marked fatty degeneration of the liver cells, so that very few cells of normal appearance could be found.

In looking over the results in Table I, obtained from the chloroformed dogs, it is seen, as in the previous cases, that arginin has become much diminished in amount. This fact is especially true of the more advanced cases (VIII and IX), where the nitrogen of arginin falls off from 55 to 60 per cent. The arginin nitrogen of the other instances (VI and VII) shows far less diminution. In the latter cases, the histidin and lysin nitrogen content seems to be somewhat in excess of the values assumed as normal, the ratios of the bases being, however, in very close agreement, as is shown by the figures 3.18 and 3.25, as compared with the normal figures of 3.33 and 3.22. The histidin and lysin values of the more advanced lesions appear within the range of normal values or slightly under them.

It is interesting to observe that in the chloroformed animals, and hence in striking contrast to the phosphorized dogs, there seems to be no abnormal falling off of the total nitrogen of the tissue examined. Thus the average total nitrogen in 100 parts of the dry liver tissue of the four chloroformed dogs is 11.32 (Table II); the corresponding average in the normal dogs is 11.41. In view of this fact, it would seem that in the chloroformed animals, particularly in the two in which there is quite a marked diminution of the nitrogen of the arginin, a transformation, rather than an absolute loss, of the nitrogen has taken place in the tissue.

In glancing over the ratios of the nine cases studied (Table I), the relation of the lysin to the histidin, with one or two exceptions, remains remarkably constant. The average of the nine ratios is 3.19.

CONCLUSION.

The results above reported lead to the conclusion that while in the degenerating cells chemical changes are taking place tending toward a diminution of the hexon bases as a whole,

they affect the arginin especially. One may picture the process either as a partial or as a complete breaking down of certain proteid material more or less rich in hexon bases, leaving behind proteid matter poorer in bases.

The meaning of these changes is, however, obscure, and with the limited number of known facts bearing upon the subject, it would seem idle even to attempt to formulate an hypothesis to explain them. Certain work of other investigators is, however, suggestive in this connection.

Kossel and Dakin,[18] for instance, as illustrated by their work upon the simple proteid body clupein, found that a partial destruction of the proteid molecule, involving the arginin group, is brought about by a ferment furnished by the animal organism. When subjected to the hydrolytic action of a mineral acid, clupein yields arginin in considerable abundance. But if the clupein is first acted upon by the ferment arginase found in the liver, and then subjected to acid hydrolysis, the yield of arginin is appreciably diminished. Among the cleavage products in the latter instance are the components of arginin, namely, ornithin and urea. It would seem, therefore, as if the ferment had loosened the union between the ornithin and urea in the arginin group, so that upon subsequent hydrolysis a diminution of arginin resulted. In the cases studied by me, it may be that the conditions were favorable for some such ferment action as that above described, and hence the relatively low yield of arginin. No attempt, however, was made to ascertain if ornithin were present in the urine. Its presence there would seem not wholly unlikely when one considers the diminished power of oxidation of the phosphorus-poisoned cell, although Thompson [19] has shown that arginin or ornithin when administered to a healthy dog as food or by hypodermic injection is eliminated for the most part as urea, no ornithin being found.

There might seem to be a conflict between this view and the results recently published by Wohlgemuth,[20] but it must be

[18] *Zeitschr. f. physiolog. Chemie*, 1904, xlii, 181.
[19] *The Journal of Physiology*, 1905, xxxii, 137.
[20] *Zeitschr. f. physiolog. Chemie*, 1905, xliv, 74.

borne in mind that the influences at work causing the breaking down of the proteid molecule are probably quite diverse in character. Wohlgemuth has recently shown for the first time that a diamino acid may actually find its way into the urine in phosphorus poisoning. He found arginin in the urine not only in rabbits poisoned with phosphorus but also in a patient suffering from phosphorus poisoning. On the other hand, he was unable to find lysin in the urine. This fact is of especial interest in view of the evidence set forth in this paper that the arginin base is lost to the proteid molecule more rapidly than the lysin base. The correspondence between the findings in the liver and in the urine is thus a close one.

How much of the arginin liberated from the proteid molecule may find its way into the urine is of course uncertain. It seems reasonable to suppose that a portion of the base is acted upon by the arginase ferment in the manner already described.

Of the seventeen to eighteen cleavage products of the proteid molecule thus far isolated, the hexon bases are among the most stable. One or more of these bases have been found in practically all proteid matter thus far investigated[21]; in fact arginin is so uniformly present that Kossel[22] has made the suggestion that it is the kernel of the proteid molecule. At all events, the question may be asked, whether, if the influences at work in the altered liver tissue were of a general character causing a diminution of the hexon bases, the monoamino acid groups would not suffer even a greater diminution; and since the pathological condition is undoubtedly associated with impaired oxidation, their presence should not be expected in the urine. As a matter of fact, Ignatowski[23] found considerable quantities of monoamino acids in the urine of patients suffering from gout, pneumonia, and leukæmia, though under normal conditions no monoamino acids were found in the urine, indeed, not even after the subcutaneous injection of glycokoll.

Furthermore, the loosening of the amino acids from the

[21] Cohnheim, *Chemie der Eiweisskörper*, pp. 42–47.
[22] *Ber. d. deutsch. chem. Gesellschaft*, 1901, xxxiv, 3214.
[23] *Zeitschr. f. physiolog. Chemie*, 1904, xlii, 371.

proteid molecule is suggested by the fact that Taylor [24] found such acids in the liver of a patient who died from a hepatic disease of obscure etiology, but which he was inclined to attribute to chloroform poisoning. Taylor found not only leucin and tyrosin in the liver, but also arginin, a fact not without interest in view of the diminished arginin content found in the livers of the chloroformed dogs after acid hydrolysis.

Moreover, the falling off of the hexon bases under the conditions studied seems quite in accordance with some results recently reported by Levene.[25] He has shown that certain cleavage products obtained by the action of mineral acids upon self-digested pancreas, spleen, and liver, are much diminished when compared with the products obtained from the fresh glands. The lysin and arginin of the digested liver, for example, showed a diminution of over 50 per cent.

It is now well established that in course of the process of aseptic autolysis, the proteids of the liver cell undergo decomposition into simpler substances, and Jacoby [26] showed that during life autolysis may go on in portions of the liver in which the circulation has been hindered. But of greater significance still in this connection, is the observation made by Jacoby on the autolytic changes in the liver during phosphorus poisoning. He found that when the normal liver substance is permitted to autolyse the solution of the liver substance is a slow one. On the other hand, under similar conditions the liver of a phosphorus-poisoned animal undergoes rapid and almost complete solution. The difference in the behavior of the normal and damaged liver points to an increase of normal ferment action in the case of the poisoned organ. It thus seems reasonable to suppose that in phosphorus poisoning we have during life an exaggerated breaking down of the proteid molecule associated with an over-action of certain ferments, and among them probably arginase.[27] The pathological

[24] University of California Publications, *Pathology*, 1904, i, 43.

[25] *The American Journal of Physiology*, 1904, xii, 276.

[26] *Zeitschr. f. physiolog. Chemie*, 1900, xxx, 174.

[27] It would appear as if the rôle of arginase in phosphorus poisoning might be advantageously subjected to experimental study. By means of the method

process in the liver during life may, therefore, be thought of as proceeding in the same general direction as the process of post-morten autolytic decomposition.

By means of further studies along lines indicated in this paper, it should be possible to gain a deeper insight into numerous pathological processes. The changes in amyloid degeneration are among those which promise to be better understood through the application of the new methods of chemical analysis. Moreover, it cannot be doubted that pharmacology as well as toxicology has much to gain from a study of what happens to the proteid molecule under the influence of poisons.

of Kossel and Dakin (*Zeitschr. f. physiolog. Chemie*, 1904, xlii, 181), it is possible to separate arginase from the liver and to measure its activity upon arginin. If the view above expressed be correct, it should be possible to determine the presence of arginase in increased amount in the liver of animals poisoned with phosphorus.

TABLE I

Division.	No. of experiment.	Weight of liver in grams.	Dry substance in 100 parts of moist liver tissue.	Nitrogen in 100 parts of dry liver tissue.	Nitrogen of bases in 100 parts of dry liver tissue:			Nitrogen of bases in percentage of total Nitrogen:			Bases in 100 parts of dry liver tissue:			Ratio of bases in liver tissue:		Remarks.
					arginin.	histidin.	lysin.	arginin.	histidin.	lysin.	arginin ($C_6H_{14}N_4O_2$) in grm.	histidin ($C_6H_9N_3O_2$)	lysin ($C_6H_{14}N_2O_2$)	*arginin.* histidin.	*lysin.* histidin.	
A. Normal.	I.	370	30.13	11.54	1.036	0.240	0.563	8.982	2.077	4.876	3.22	0.88	2.93	3.66	3.33	Normal dog. Killed by bleeding.
	II.	435	28.50	11.28	1.156	0.271	0.618	10.25	2.407	5.483	3.59	1.00	3.22	3.59	3.22	Normal dog. Killed by bleeding.
	III.	180	22.59	12.48	1.088	0.298	0.488	8.720	2.388	3.912	3.38	1.10	2.55	3.07	2.32	Normal dog. Liver washed.
B. Phosphorus.	IV.	194	26.74	7.34	0.370	0.133	0.284	5.046	1.809	3.868	1.15	0.490	1.48	2.35	3.02	Phosphorus dog. Killed by bleeding.
	V.	260	21.90	7.90	0.327	0.099	0.214	4.143	1.256	2.710	1.02	0.366	1.12	2.79	3.06	Phosphorus dog. Liver washed.
C. Chloroform.	VI.	340	24.12	11.96	0.762	0.371	0.832	6.369	3.103	6.961	2.37	1.37	4.34	1.73	3.18	Chloroform dog. Killed by bleeding.
	VII.	275	24.66	11.32	0.785	0.324	0.746	6.935	2.862	6.592	2.44	1.20	3.89	2.04	3.25	Chloroform dog. Killed by bleeding.
	VIII.	—	22.47	11.49	0.436	0.193	0.531	3.798	1.678	4.624	1.35	0.712	2.77	1.90	3.89	Chloroform dog. Prolonged treatment. Killed by bleeding.
	IX.	—	31.33	10.51	0.458	0.224	0.540	4.360	2.133	5.139	1.42	0.827	2.82	1.72	3.41	Chloroform dog. Prolonged treatment. Found dead.

TABLE II

(1) Division.		(2) No. of experiment.	(3) Dry substance in 100 parts of moist liver tissue.	(4) Nitrogen in 100 parts of dry liver tissue.	(5) Nitrogen in percentage of total Nitrogen: arginin.	histidin.	lysin.	the three bases.	(6) Bases in 100 parts of dry liver tissue: arginin.	histidin.	lysin.	the three bases.
A. Nor.		Average I. II.	29.32	11.41	9.616	2.242	5.180	17.04	3.41	0.94	3.08	7.44
B. Phos.		IV.	26.74	7.34	5.046	1.809	3.868	10.72	1.15	0.49	1.48	3.12
C. Chlor.		Average VI. VII. VIII. IX.	25.65	11.32	5.366	2.444	5.829	13.64	1.90	1.027	3.45	6.38
Washed liver.	Nor.	III.	22.59	12.48	8.720	2.388	3.912	15.02	3.38	1.10	2.55	7.03
	Phos.	V.	21.90	7.90	4.143	1.256	2.710	8.11	1.02	0.366	1.12	2.51

TABLE III

Division.	No. of experiment.	Diminution of Nitrogen in percentage of corresponding normal nitrogen values.[1]					Diminution of bases in percentage of corresponding normal values in dry liver tissue[2]:			
		Nitrogen in 100 parts of dry liver tissue.	Nitrogen of arginin.	Nitrogen of histidin.	Nitrogen of lysin	Nitrogen of the three bases.	arginin.	histidin.	lysin.	the three bases.
B. Phos.	IV.	35.67	47.52	19.32	25.33	37.10	66.27	47.87	51.94	58.06
	V. Liver washed.	36.70	52.49	47.41	30.73	46.01	69.82	66.73	56.08	64.28
C. Chlor.	Average VI. VII. VIII. IX.	0.79	44.20	9.01[3]	12.53[3]	2.00	44.28	9.26[3]	12.01[3]	0.81

[1] Calculated from columns 4 and 5, Table II.
[2] " " column 6, Table II.
[3] Increase.

THE CONSTRUCTION OF A THERMOSTAT-ROOM.

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Plate XXVII.

The receipt by the author of a number of inquiries regarding the building of a thermostat-room has led him to bring together in a brief paper certain facts which might prove of value to those interested in the subject.

Every well-appointed bacteriological laboratory, in which teaching of large-sized classes is carried on, should be equipped with an incubator-room, for experience has shown that the costly copper thermostat, even of largest size, is entirely inadequate to afford proper service, and, therefore, the question resolves itself into one of two alternatives, namely, that of furnishing two or more ordinary thermostats at a considerable expense, or of erecting a thermostat-room at a much less price and with the additional advantage of better service.

After having seen in 1901 a rather rudimentary thermostat-room in Copenhagen, Denmark, and having had a hurried and unsatisfactory glimpse of a thermostat-room in the Institute for Infectious Diseases in Berlin, plans were prepared to suit the conditions found in the pathological laboratory of the Johns Hopkins University, where a very limited floor-space was the chief obstacle in the way of the erection of an ideally constructed room. The room had, furthermore, to be built independently of the walls of the laboratory, as these formed the outer corner-walls of the building, and the plastering inside was placed directly on the bricks, thus compelling the abandonment of their use in forming the walls of the incubator, since this construction would have resulted in too great and constant absorption of heat.

The framework of the incubator-room consisted of moderately heavy pine scantling, properly joined and covered on the outside with three-quarter inch dressed and matched white-pine boards, and on the inside with rough one-inch boards, enclosing a space six inches in width between on all sides, except beneath the floor, where the space was three inches, thus forming an apartment whose inside measurements were 8 feet high, 5.5 feet wide, and 4.5 feet deep. A door 6 feet high and 24 inches wide, having the same construction as the walls, opened outwards on the longer wall at one end, and an inner sliding door with a glass window was provided to prevent the loss of heat when the outer door was opened. The whole of the inside walls and ceiling was covered with carefully matched linoleum, which was afterwards varnished. Ventilation was provided for by two apertures two inches in diameter, one being placed three inches above the floor in one corner of the room and protected against the entrance of rats and mice by a wire grating, the other in the centre of the ceiling, both being provided with sliding valves to permit of regulation of the change of air.

Seven rows of shelving, approximating 56 feet in length, made of one-inch dressed pine, 12 inches wide, were installed on the side opposite to and on the end farthest from the entrance.

The room was lighted by a 16 candle-power incandescent electric lamp on a wire carried in through the ceiling ventilator, and controlled by a switch placed outside on the lintel of the door.

The heating was the main problem connected with the design, and after much consideration hot water was decided upon as the most efficient medium to be employed. The apparatus consisted of a ten-gallon galvanized iron boiler placed on an iron stand some eight or nine inches high. From the centre of the upper surface of the boiler one-inch piping was carried into the room at the end farthest removed from the entrance, and back and forth down the side and along three-quarters of the length of the opposite end of the room, forming four rows of piping, comprising a total length of 48 feet, the lowermost end passing outside on a plane level with the lower end of the boiler with

which it connected (Fig. 1). An air valve with cock was affixed to the outflow pipe immediately where it leaves the boiler, being carried upwards some three or four inches to trap effectually the air which might have accumulated during the filling of the pipes. A cock was affixed to the return flow pipe near the boiler for the purpose of emptying the system when necessary.

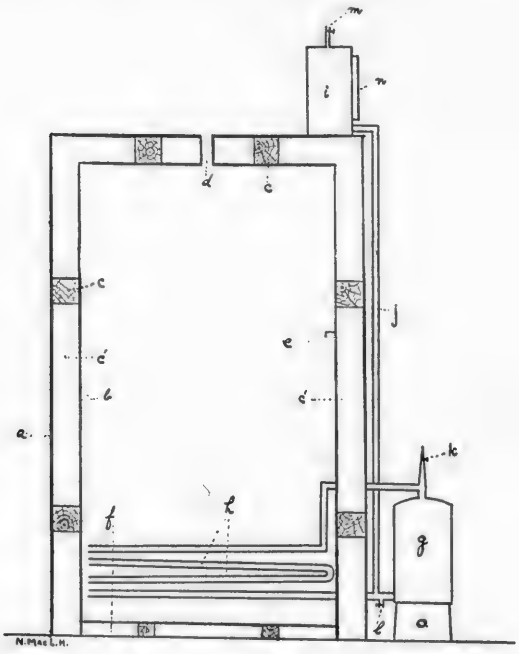

FIG. 1.

End Sectional View of Thermostat-Room and Heating Apparatus.
(Scale $\frac{1}{2}$ in. to 1.5 ft., approximately.)

a. Outer wall of matched pine boards. b. Inner wall of undressed pine boards. c. Framework of scantling. c^1. 6 in. interspace filled with slag wool. d. 2 in. ventilator in ceiling. e. Shelf for thermoregulator. f. Interspace 3 in., like c^1. g. Boiler, 10-gallon capacity, 20 x 12 in. h. Radiating pipe, 1 in. i. Expansion tank, 5-gallon capacity, 18 x 9 in. j. Pipe, $\frac{3}{4}$ in., connecting tank with boiler. k. Air-cock. l. Water-cock for draining heating system, or for filling it. m. Air or water-cock. n. Gauge. o. Space below boiler (enclosed) for Koch safety-burner.

Since the thermostat-room was on the same level as the floor of the laboratory, and it was desired to keep the heating surfaces as low down as possible, and as the boiler outlet had then unavoidably to be on a plane higher by twelve inches than the desired level of the uppermost line of piping inside, the difficulty of securing proper circulation was met with at the outset. As it was impossible to install the boiler in the room below, the following device was resorted to in order to supply *vis a tergo:* a five-gallon expansion tank fitted with a glass water-gauge was erected on the roof of the incubator directly over the boiler, at a height of about ten feet, connection being made with the return flow pipe close to the boiler by means of three-quarter inch piping (Fig. 1). This device proved entirely satisfactory, the resistance which might have been offered, caused by the sudden drop of twelve inches in the outflow level, being entirely overcome and the circulation being quite rapid.

The system was filled by carrying a line of hose to a cock on the upper surface of the expansion tank, but it might have been done equally well by making a similar connection with the exhaust cock below.

The gas supply entered the room from above through a half-inch pipe connecting with and passing through a Lothar-Meyer ether-mercury regulator, thence by means of quarter-inch lead pipe to a large-sized Koch safety-burner placed beneath the boiler. The temperature was readily enough maintained at 37° C., except during very cold weather, when it fell to 34° C. This fall was explained by reason of the fact that the steam heat supplied to the laboratory was always cut off at night, and the boiler having been placed immediately below a large window readily lost a considerable amount of heat by radiation. This last drawback could probably be overcome by plastering the boiler all over with asbestos plaster to the depth of one inch.

The cost of the completed room, exclusive of the thermo-regulator and the Koch safety-burner, was one hundred and twenty dollars, which sum is often exceeded by the cost of the larger sizes of the ordinary copper-built apparatus.

Naturally enough the incubator did not prove perfect, and

after it had stood the test of a session's work certain imperfections and shortcomings were noted, to which brief consideration will be given.

In the first few weeks after starting the apparatus, considerable difficulty was experienced by reason of the air accumulating in that portion of the outflow pipe at or near the downward bend inside the room (Fig. 1), causing a stoppage of the circulation. This difficulty could be remedied by bringing the pipe into the room on a slight slope before dropping it down, thus permitting the air to flow upwards to the air valve without any hindrance.

The plastering of the boiler has already been mentioned as a desirable improvement.

It would be an advantage to place the boiler at a lower level than the pipes, not indeed by carrying the pipes higher up on the wall, but by placing the boiler on a lower floor of the building directly beneath the incubator. Still other means of accomplishing this end might, however, suggest themselves.

A more satisfactory gas regulator than the Lothar-Meyer is the Roux bi-metallic regulator, as it rarely requires attention and hence it could be substituted with advantage in the construction of the room.

Automatic lighting of the room would be a further improvement. This could be accomplished by some style of door connection whereby the light would be turned on upon opening, and shut off on closing, the outer door.

The shelving might be replaced advantageously by the erection of a series of individual drawers, constructed so as to permit of the free circulation of the heated air of the room among them.

A vestibule with swinging door would be a benefit, since it would prevent too great loss of the warm air of the room on the opening of the thermostat door.

In presenting this paper it is the writer's intention to suggest a plan which applies to those cases only in which facilities for making alterations in a building to meet more properly the demands of a large bacteriological class cannot be carried out. Where a new building is being erected, or alterations are being carried out on an old one, the scheme as outlined in the fore-

going description can doubtless be rendered more simple and less expensive.

ADDENDUM.

Since the foregoing paper was presented, I have had the privilege of inspecting a thermostat of much smaller dimensions but built on similar lines to the one above described. It is in the laboratory of the Thomas Wilson Sanitarium for Sick Children at Mount Wilson, Md., and was made from the designs of Dr. V. H. Bassett of the Johns Hopkins University Medical School, to whose kindness I am much indebted for the accompanying diagram and the permission to make use of the data of construction.

The thermostat was set up on the work-bench in one corner of the laboratory, and was in use during the summers of 1903 and 1904, and gave entire satisfaction.

Its inside measurements were 106 cm. high, 72 cm. wide, and 72 cm. deep, giving a capacity of 0.55 cb.m. It was built upon a wooden framework, using on the inside one-half inch dressed pine boards, afterwards covered by linoleum, whilst the outside was covered by ordinary wainscoting; the inner and outer walls inclosing a space of 6 cm. wide which was packed with cork shavings (Fig. 2). Three movable wooden shelves were set up 20 cm. apart. Holes 2.5 cm. in diameter were bored upon the top to permit ventilation, and to take a thermoregulator and a thermometer; a hole of similar size, for the inlet of air, was made on one side low down. Two doors were provided, an inner and an outer, equal in height and width to the measurements of one side of the apparatus. The door-jamb was faced with felt all around so that the inner door (which was covered on its internal surface with linoleum) might fit closely.

Hot water being the medium employed to furnish heat, the necessary apparatus for such a system consisted of a boiler, circulating pipes, and expansion tank. The boiler was made of galvanized sheet-iron soldered together, measuring 26 x 17 cm., with a capacity close to six litres, and placed upon a covered stand on the floor of the room beneath the thermostat.

Running from it and leading into the incubator was a galvanized iron pipe, having an inside diameter of 1.25 cm., and disposed in eleven rows along the back wall of the incubator

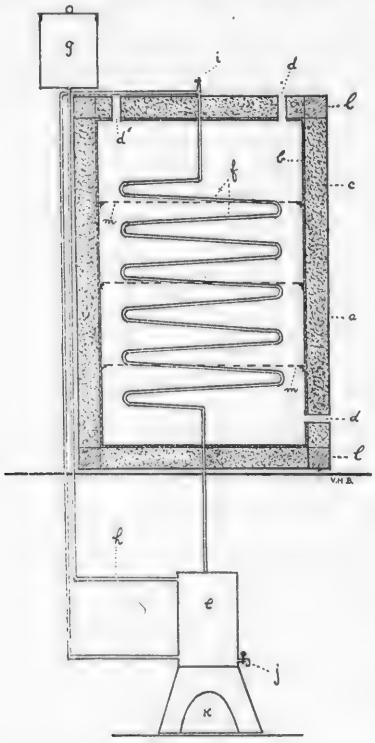

FIG. 2.

End Sectional View of Dr. Bassett's Thermostat (scale about $\frac{1}{20}$).

a. Outer wall of matched pine boards. b. Inner wall of dressed pine. c. 6 cm. interspace filled with cork shavings. d. 2.5 cm. ventilation shaft. d^1. Shaft for thermoregulator. e. Boiler, 17 x 26 cm., capacity 6 litres. f. Radiating pipe. g. Expansion tank, 20 x 16 cm., capacity 4 litres. h. Pipe, connecting tank with boiler. i. Air-cock. j. Water-cock. k. Space below boiler for Koch safety-burner. l. Wooden frames. m. Position of shelving.

in zig-zag fashion; as it passed out it was capped with an air-cock, after which it continued downwards to the lower part of the boiler. The piping gave, approximately, 4140 square cm.

FIG. 3.

Photograph of Dr. Bassett's thermostat in the Thomas Wilson Sanitarium for Sick Children.

of radiating surface. The expansion tank consisted also of galvanized iron soldered together and provided with a cover; it was connected to the upper parts of the boiler and measured 20 x 16 cm., and had a capacity of about four litres.

The thermoregulator was of the Reichert type and the boiler was heated by a Koch safety-burner of ordinary size.

The actual cost of materials, some of which were lying ready at hand, was $11.00, a workman's wages was $7.00, making a total cost of $18.00; if made by contract, the price doubtless would approximate $25.00.

ENZYMES AND ANTI-ENZYMES OF INFLAMMATORY EXUDATES.

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Many substances insoluble in the fluids of the body when injected into the tissues undergo solution through the agency of phagocytic cells which surround or engulf them. Much study has been devoted to the factors which influence phagocytosis but comparatively little attention has been given to the process of intracellular digestion.

The occurrence of pepton in pus was demonstrated by Eichwald [1] as early as 1864, and later confirmed by Maixner [2] and Hofmeister.[3] In association with a variety of diseases characterized by formation and accumulation of pus, such as empyæma, purulent peritonitis, abscess, epidemic cerebro-spinal meningitis, and pyelitis, Maixner found pepton in the urine. Pepton was also observed in the urine during the resolution of croupous pneumonia. Finding that the amount of pepton present in purulent fluid bore a relation to the number of cells which were present, and was more abundant in the cellular sediment than in the overlying serum or in the filtrate from such an exudate, Hofmeister reached the conclusion that living pus cells have the power of chemically or mechanically binding pepton, and peptonuria is a symptom of the destruction of pus cells.

To explain the constant presence of pepton in purulent sputum, Kossel [4] cites hitherto apparently unpublished observations of Fr. Müller, who had found that a glycerine extract of purulent

[1] *Würzb. med. Zeit.*, 1864, 335. Cited by Hofmeister.
[2] *Prager Vierteljahrschrift*, cxliii, 78. Cited by Hofmeister.
[3] *Zeit. für phys. Chem.*, 1880, iv, 253, 268.
[4] *Zeit. für klin. Med.*, 1888, xiii, 149.

phthisical sputum is capable of digesting in a weakly alkaline medium fibrin and coagulated albumin if decomposition is prevented. Other purulent sputa have the same power, but purely mucous sputum and the sputum of pneumonia before the crisis do not exhibit it. As soon as crisis occurs and the sputum has assumed a white pus-like appearance, the presence of a ferment is demonstrable. In the fresh pus obtained from an abscess, a similar ferment can be demonstrated, but is absent in the thin pus-like fluid of a cold abscess. Achalme [5] has studied the effect of pus upon gelatin, fibrin, coagulated egg-albumin, and casein, and has found that it is capable of liquefying or dissolving these substances. Similar observations have been made by Leber.[6]

Ascoli and Mareschi [7] subjected the cellular sediment of sterile exudates, obtained by injecting aleuronat into the peritoneal cavity, to autolysis under chloroform, and determined before and after digestion, by means of the Kjeldahl method, the amount of nitrogen not precipitable by tannin. A well marked digestion occurred but was absent when the cellular sediment had been previously heated to 60° C. for three hours.

Attempts to study the autolysis of fluids withdrawn from the serous cavities of individuals suffering with peritoneal or pleural effusions have given inconstant results. Estimating by means of the Kjeldahl method the amount of proteid converted by digestion into non-coagulable nitrogen-containing substances, Umber [8] showed that the peritoneal fluid from two patients suffering with abdominal tumors underwent autolysis. In ascitic fluid from five patients and in pleural exudates from two, Schütz [9] found by the same method no measurable destruction of proteids. The fluid in two instances contained a considerable number of leucocytes. The autolysis of exudates withdrawn from serous cavities of twelve patients suffering with hydro-

[5] *Compt. rend. de la Soc. de Biol.*, 1899, li, 568.

[6] *Ueber die Entstehung der Entzündung*, Leipzig, 1891.

[7] *Eleventh Italian Congress for Internal Medicine*, Pisa, October 27–31, 1901. Ref. Maly's *Jahresber.*, 1902, xxxii, 291.

[8] *Münchener med. Woch.*, 1902, xlix, 1169.

[9] *Cent. für inner. Med.*, 1902, xxiii, 1161.

thorax, pleurisy, and ascites has been studied by Zak.[10] In one-half of the cases, including two cases of pleurisy, no autolysis was demonstrable, while in only four instances was there noteworthy digestion of coagulable albumin. Zak found no relation between the abundance of cells and the degree of autolysis.

Methods.—The present series of experiments was undertaken with the purpose of studying the capacity for proteolytic digestion possessed by inflammatory exudates at various stages. Exudates, obtained by injecting suspensions of plasmon or of aleuronat into the pleural cavities of dogs or rabbits, were, with aseptic precautions, removed from the animal killed by bleeding or were withdrawn from the etherized animal by aspiration. A suspension of plasmon in ten times its weight of 0.5 % potassium hydroxide was at first used, but aleuronat meal in from seven to ten times its weight of water, to which was added 3 % of starch, proved more useful. Of this suspension 10 cubic centimeters were injected into a pleural cavity. The exudate removed at the end of from one to three days was introduced in carefully measured quantities into small flasks and diluted with four times its volume of 0.85 % salt solution. In most instances 1 cubic centimeter of toluol was added to each flask, which was then placed in the thermostat at 37° C. At the end of two or more days the flasks were removed and their contents were heated to 100° C., after acidification with acetic acid.

Complete precipitation of the coagulable proteids was at first effected with some difficulty, but was later much facilitated by the addition of an equal volume of 16 % solution of magnesium sulphate. Should precipitation be incomplete, filtration is difficult and the filtrate is turbid. By adjusting the reaction to slight acidity with acetic acid (1 %), or with sodium hydroxide ($\frac{1}{10}$ N.) if acid has been added in excess, albumin still in solution may be coagulated by heat and again removed by filtration. The filtrate from the coagulum repeatedly washed was received directly into a Kjeldahl flask and the amount of nitrogen present was estimated by the Kjeldahl method.

[10] *Wiener klin. Woch.*, 1905, xviii, 376.

The filtrate from an equal amount of exudate diluted in the same way and immediately coagulated by heat served as a control. The difference between the autolyzed exudate and its control represents in terms of $\frac{1}{10}$ N. sulphuric acid the nitrogen of the coagulable proteid converted by digestion into soluble form. I am indebted to Dr. Flexner and to Dr. P. A Levene for numerous suggestions which have aided me in the application of the chemical methods employed.

Autolysis of Sterile Inflammatory Exudates.—In the following experiments, sterile exudates, obtained by injecting a suspension of aleuronat into the right pleural cavity of dogs, were removed aseptically and kept in sterile flasks without addition of toluol or other antiseptic for forty-eight hours at body temperature. That no autolysis had occurred during this time is shown by the following figures which represent in terms of $\frac{1}{10}$ N. acid the nitrogen of substances in solution after coagulation by heat.

Experiment 1.—Reddish turbid fluid containing many leucocytes and a considerable number of red blood corpuscles was removed from the right pleural cavity of a dog twenty-four hours after the injection of aleuronat. Nitrogen present in the filtrates from the coagulated fluid is represented by c. c. of $\frac{1}{10}$ N. acid.

5 c. c. exudate at 37° C. 2 days	3.1 c. c.
Control	3.7 "

Experiment 2.—Exudate was removed forty-eight hours after the injection of aleuronat into the right pleural cavity of a dog. The exudate contained 6.5 % of cells.

5 c. c. exudate at 37° C. 2 days	3.05 c. c.
Control	3.75 "

In both experiments less nitrogen was found in the control subjected to immediate coagulation than in the flask kept forty-eight hours at body temperature. The significance of this fact, confirmed by subsequent experiments, will be discussed later.

In similar experiments the exudates obtained from rabbits by injecting aleuronat into the pleural cavity were used. The diluted exudate was kept at body temperature four days.

Experiment 3.—Exudate was removed twenty-four hours after the injection of 10 c. c. of aleuronat suspension into the right pleural cavity of a rabbit. The cells present formed 6 % of its volume.

3 c. c. exudate at 37° C. 4 days (aseptic)	0.7 c. c.
Control	0.9 "

EXPERIMENT 4.—Exudate was removed two days after the injection of 5 c. c. of aleuronat suspension. Cells form 5.5 % of the volume.

3 c. c. exudate at 37° C. 4 days (with toluol)	1.65 c. c.
Control	1.7 "

EXPERIMENT 5.—Exudate was removed three days after the injection of 10 c. c. of aleuronat. Cells form 2.5 % of the volume.

1 c. c. exudate at 37° C. 4 days (aseptic)	2.10 c. c.
Control	1.55 "

No autolysis was obtained except with the exudate removed three days after the injection of the inflammatory irritant. Since it was suspected that autolysis occurred only after the death of cells contained in the exudate, longer periods of digestion were considered necessary, and at the same time the effect of toluol, which presumably destroys the cells, was studied. A considerable number of flasks containing equal quantities of exudate, some aseptically prepared, others prepared with toluol, were kept at body temperature for varying periods as indicated below.

EXPERIMENT 6.—An exudate containing 4 % cells was obtained twenty-four hours after injecting 10 c. c. aleuronat suspension into the right pleural cavity of a dog. Each flask contained 3 c. c. exudate diluted as in previous experiments.

	Aseptic.	With toluol.
Control	2.0 c. c.	
At 37° C. 2 days	2.0 "	1.3 c. c.
" " " 4 "	2.1 "	2.15 "
" " " 6 "	1.7 "	2.5 "
" " " 8 "	1.7 "	2.55 "

EXPERIMENT 7.—An exudate containing 2.5 % cells was removed from the right pleural cavity of a dog three days after the injection of 10 c. c. aleuronat. Each flask contained 3 c. c. exudate.

	Aseptic.	With toluol.
Control	7.9 c. c.	7.7 c. c.
At 37° C. 2 days	7.8 "	7.7 "
" " " 4 "	9.9 "	10.25 "
" " " 6 "	12.9 "	10.9 "

Exudates removed both from the dog and from the rabbit one or two days after the injection of aleuronat underwent little if any digestion, while that removed three days after injection autolyzed to an appreciable though slight extent. The presence of toluol did not very materially affect the result in either case, and greatly facilitates the performance of the experiment, since

it obviates the necessity of preserving the strictest asepsis when, as in subsequent experiments, centrifugalization of the exudate is necessary. In the preceding experiments, cultures made from each flask at the end of digestion remained sterile.

It is noteworthy that Schütz, attempting to autolyze exudates obtained by tapping the pleural or peritoneal cavities, obtained negative results, while those of Zak were inconstant. Zak found no relation between autolysis and the cellular contents of the exudate.

Autolysis of Leucocytes.—In several instances the quantity of cells in the exudates employed in the preceding experiments was estimated by means of the hæmatocrit and varied by volume from 2.5 % to 6.5 %. There was no relation between the proportion of cells and the occurrence of autolysis. In the exudates obtained from rabbits, those removed one and two days after injection contained respectively 6 % and 5.5 % of cells, while the exudate which was removed on the third day and which underwent slight digestion contained 2.5 %. In order to determine if autolysis affected only the cells, in the following experiments cells of an exudate twenty-four hours after injection of aleuronat were separated from the serum by centrifugalization and suspended in nineteen times their volume of .85 % solution of sodium chloride (5 % suspension). The suspension was diluted with four times its volume of salt solution and subjected to autolysis during five days. An equal amount of serum was kept during the same time at 37° C. and compared with a control flask coagulated immediately.

EXPERIMENT 8.—In 5 c. c. of a 5 % suspension of cells and in 5 c. c. of serum, obtained from an exudate removed twenty-four hours after the injection of aleuronat into the pleural cavities of a dog, the amount of nitrogen contained in substances not coagulable by heat before and after digestion is represented in terms of $\frac{1}{10}$ N. acid by the following figures:

5 c. c. cells at 37° C. 5 days	2.35 c. c.
Control	.75 "
5 c. c. serum at 37° C. 5 days	3.7 "
Control	4.15 "

With cells alone well marked autolysis occurred, although with the entire exudate removed at the same stage of inflam-

mation and containing approximately the same number of cells, none was demonstrable (e.g., Experiment 2, in which the percentage of cells in the exudate used was 6 %). It is noteworthy that the serum kept at 37° C. for five days, like the whole exudate in previous experiments, contained less nitrogen in soluble form than the control coagulated immediately.

Anti-enzymotic Action of the Serum of Sterile Inflammatory Exudates.—The experiment just described has suggested that the serum of the inflammatory exudate inhibits the autolysis of the leucocytes which it contains. Obviously this possibility may be tested by separating, by centrifugalization, cells and serum and digesting separately and after recombining.

In the following experiment, cells from an exudate removed from the pleural cavity twenty-four hours after the injection of aleuronat were suspended in nine times their volume of salt solution. In order to test the autolysis of these cells, 5 cubic centimeters of the suspension were diluted with 20 cubic centimeters of salt solution. To the same quantity of cells was added 1 cubic centimeter, and in another flask 5 cubic centimeters, of the serum of the exudate, and in every instance the total volume of fluid was brought to 25 cubic centimeters by the addition of salt solution. In another flask 5 cubic centimeters of serum, diluted with 5 cubic centimeters of salt solution, were heated for twenty minutes in boiling water; to this coagulated serum were added 5 cubic centimeters of the suspension of cells. After addition of toluol the various mixtures were kept at 37° C. for five days. The nitrogen in uncoagulable compounds, contained in 5 cubic centimeters of serum from the exudate diluted to 25 cubic centimeters and allowed to remain in the thermostat for the same time, was, for the sake of comparison, estimated by the usual method.

EXPERIMENT 9.—Cells and serum, obtained from an exudate removed from the pleural cavities of a dog twenty-four hours after the injection of aleuronat, were tested as follows:

5 c. c. cells at 37° C. 5 days	8.2	c. c.
Control	1.3	"
Digestion	6.9	"

5 c. c. serum at 37° C. 5 days	4.35 c. c.
5 c. c. cells + 1 c. c. serum at 37° C. 5 days	3.45 "
Uncoagulable nitrogen in 5 c. c. cells (1.3 c. c.) and 1 c. c. serum (approximately 0.87 c. c.) represented by	2.17 "
Digestion	1.28 "
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	7.75 " *
Uncoagulable nitrogen in 5 c. c. cells (1.3 c. c.) and 5 c. c. serum (approximately 4.35 c. c.) represented by	5.65 "
Digestion	2.1 "
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	30.65 "
Uncoagulable nitrogen in 5. c. c cells and 5 c. c. serum represented by	5.65 "
Digestion	25.00 "

* Culture from this flask showed bacterial contamination; the flask had been insecurely closed and the toluol used had escaped.

The autolysis of the leucocytes contained in the suspension is inhibited by the addition of serum, so that the nitrogen derived by digestion from coagulable proteids is diminished to less than one-third. When, however, the serum is heated to approximately 100° C. no inhibition occurs and digestion proceeds far beyond that referable to autolysis of cells. The intracellular ferments, the action of which is now unrestrained, attacks the proteids furnished by the heated serum.

The following experiments confirm the results just described:

Experiment 10.—Cells obtained twenty-four hours after the injection of aleuronat into the pleural cavities of a dog were suspended in nineteen times their volume of salt solution.

5 c. c. cells at 37° C. 5 days	4.55 c. c.
Control	2.05 "
Digestion	2.5 "
5 c. c. serum at 37° C. 5 days	3.85 "
Control (coagulated immediately)	4.5 "
Digestion	——
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	5.4 "
Uncoagulable nitrogen in 5 c. c. cells (2.05 c. c.) and 5 c. c. serum (4.5 c. c.) represented by	6.55 "
Digestion	——

5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days....	14.2	c. c.
Uncoagulable nitrogen in 5 c. c. cells and 5 c. c. serum represented by.....	6.55	"
Digestion.....	7.65	"

EXPERIMENT 11.—Cells from an exudate removed from the pleural cavities of a dog three days after the injection of aleuronat were suspended in nine times their volume of salt solution.

5 c. c. cells at 37° C. 5 days.....	9.3	c. c.
Control.....	3.6	"
Digestion.....	5.7	"
5 c. c. serum at 37° C. 5 days.....	6.5	"
Control (coagulated immediately).....	7.25	"
Digestion.....	——	
5 c. c. cells + 1 c. c. serum at 37° C. 5 days.....	10.2	"
Uncoagulable nitrogen in 5 c. c. cells (3.6 c. c.) and 1 c. c. serum (1.45 c. c.) represented by.....	5.05	"
Digestion.....	5.15	"
5 c. c. cells + 1 c. c. coagulated serum at 37° C. 5 days.....	11.05	"
Uncoagulable nitrogen in 5 c. c. cells and 1 c. c. serum represented by.....	5.05	"
Digestion.....	6.00	"
5 c. c. cells + 5 c. c. serum at 37° C. 5 days.....	10.95	"
Uncoagulable nitrogen in 5 c. c. cells (3.6 c. c.) and 5 c. c. serum (7.25 c. c.) represented by	10.85	"
Digestion.....	.1	"
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	23.1	"
Uncoagulable nitrogen in 5 c. c. cells and 5 c. c. serum represented by.....	10.85	"
Digestion.....	12.25	"

It is noteworthy that the inhibitory action of the serum of an exudate used in the last experiment and removed three days after the injection of the imflammatory irritant is less than that of the serum from the exudates employed in the two preceding experiments and withdrawn only twenty-four hours after injection. One cubic centimeter of the serum in this experiment had little effect upon the digestion of the cells mixed with it (digestion of cells alone, 5.7 cubic centimeters; of cells with 1

cubic centimeter serum, 5.15 cubic centimeters), while in the preceding experiments the same amount of serum completely prevented or materially retarded digestion. This observation is in agreement with the fact already noted, that the whole exudate removed one or two days after the onset of inflammation undergoes little if any autolysis, while that of exudates three days old is considerable.

That the digestion of other proteids by enzymes contained in the leucocytes is inhibited by the serum of the exudate has been shown by several readily performed experiments in which gelatin and fibrin have been subjected to the action of cells and to cells with serum.

EXPERIMENT 12.—To test-tubes containing 6 c. c. 6 % gelatin was added 1 c. c. 20 % suspension of cells from an exudate obtained twenty-four hours after the injection of aleuronat. To a second gelatin tube were added 1 c. c. suspension of cells and 1 c. c. serum from the same exudate. After remaining twenty hours at 37° C., the gelatin containing cells alone remained liquid after cooling while that with cells and serum solidified.

EXPERIMENT 13.—To one of two tubes containing balanced quantities of fibrin obtained from dog's blood and cut into small pieces, were added 2 c. c. 20 % suspension of cells from an exudate removed two days after injection of aleuronat; to the second, 2 c. c. suspension and 2 c. c. serum from the exudate, the volume being made in both instances 8 c. c. with salt solution. At the end of forty-eight hours under toluol at 37° C. fibrin in the tube containing cells had almost completely disappeared, while that mixed with cells and serum was apparently unchanged.

Effect of Temperature upon the Anti-enzymotic Action of the Exuded Serum.—It has already been shown that the anti-enzymotic action exerted by the serum of a sterile exudate upon the proteolytic ferment of the leucocytes is destroyed by a temperature of 100° C. In order to determine at what point of an ascending scale the inhibitory power is destroyed, equal quantities of serum, diluted with an equal volume of salt solution, were heated to 45°, 55°, 65°, 75°, and 100° C. for one-half hour and then mixed with a suspension of cells, the entire mixture being brought to a volume of 25 cubic centimeters by addition of salt solution.

EXPERIMENT 14.—Cells from an exudate obtained three days after the injection of aleuronat into the pleural cavity of a dog were, after repeated washing

suspended in nine times their volume of salt solution. Mixtures prepared as follows were kept in the thermostat at 37° C. for five days:

(5 c. c. cells	+	5 c. c. serum)	coagulated as control					8.45	c. c.
" "	+	"						9.9	"
" "	+	"	"	heated to	45°	C.		8.5	"
" "	+	"	"	"	55°	C.		8.8	"
" "	+	"	"	"	65°	C.		7.8	"
" "	+	"	"	"	75°	C.		25.05	"
" "	+	"	"	"	100°	C.		27.0	"

A temperature of 75° C. evidently suffices to destroy the anti-enzymotic action of the serum. Lower temperatures apparently cause a slight increase of the inhibition exerted by the serum, but the explanation of the slight change which occurs is not clear. It is not improbable that a temperature lower than 75° C. produces changes in the serum which exert an unfavorable influence upon the ferment or ferments contained in the leucocytes, or, as will subsequently appear, upon a proleolytic enzyme contained in the serum and destroyed perhaps by 65° C.

Effect of Reaction upon the Enzymes and Anti-enzymes of Sterile Inflammatory Exudates.—It was next considered desirable to study the influence of reaction upon this anti-enzymotic action of the serum of sterile exudates. This exuded serum is strongly alkaline to litmus. To mixtures of cells and serum, prepared as in previous experiments, was added sodium bicarbonate or acetic acid to the strength of 0.2%. For comparison the same amount of alkali or acid was added to similar mixtures of cells and coagulated serum.

EXPERIMENT 15.—Cells from an exudate removed twenty-four hours after the injection of aleuronat were suspended in nine times their volume of salt solution. Mixtures containing 5 c. c. of this suspension and 5 c. c. of serum were kept in the thermostat at 37° C. for five days.

	Cells + serum.	Cells + coagulated serum.
With 0.2 % sodium bicarbonate	10.1 c. c.	35.05 c. c.
Reaction unchanged	12.9 "	24.0 "
With 0.2 % acetic acid	27.05 "	19.55 "

The leucocytes of the exudate are capable of digesting proteids in both an alkaline and in an acid medium, but are more efficient in the former. The inhibition exerted by the serum

is favored by an increase of the normal alkalinity of this fluid but is completely lost with the acidity produced by 0.2 % acetic acid. In this acid medium there is, indeed, more digestion of proteid with unheated than with heated serum. The possibility that the serum of the exudate contains an enzyme active in an acid medium is suggested. The preceding experiment was repeated and at the same time to serum alone was added alkali or acid in the quantity previously used.

EXPERIMENT 16.—Cells obtained from an exudate three days after the injection of aleuronat were suspended in nine times their volume of salt solution. Mixtures containing 5 c. c. suspension of cells and 5 c. c. serum, 5 c. c. suspension and 5 c. c. coagulated serum, and 5 c. c. serum alone were prepared as follows and kept in the thermostat at 37° C. for five days:

	Cells + serum.	Cells + coagulated serum.	Serum.
With 0.2 % sodium bicarbonate..	8.0 c. c.	35.15 c. c.	6.25 c. c.
Reaction unchanged.....	9.9 "	27.0 "	4.6 "
With 0.2 % acetic acid.....	33.8 "	26.3 "	13.75 "

The nitrogen of uncoagulable substances contained in 5 c. c. of the serum before digestion is represented by 6.3 c. c. $\frac{1}{10}$ N. acid.

The experiment confirms the results previously obtained. An increased alkalinity favors the inhibition of the intracellular ferments, which are, when this inhibition is destroyed by heat, themselves most active in an alkaline medium. The addition of acid to a mixture of unheated serum and cells destroys the anti-enzymotic action of the serum, but in this acid medium digestion is more active than in a mixture of cells and serum of which the anti-enzymotic action has been destroyed by heat. This increased digestion is doubtless due to the action of a ferment contained in the serum and made active by the addition of acetic acid. For no proteolysis occurs when alkali is added to serum alone, and, when the reaction is unchanged, the same diminution of nitrogen in uncoagulable form, noted in preceding experiments, is found, but in an acid medium there is well marked digestion of proteid (represented by 13.75 minus 6.3 equals 7.45 cubic centimeters). The serum contains a ferment which, being destroyed by heat, explains the greater proteolysis obtained with cells and unheated serum than with

cells and heated serum (represented by 33.8 minus 26.3 equals 7.5 cubic centimeters).

In the serum of the blood Delezenne and Pozerski [11] have demonstrated the existence of a proteolytic enzyme capable of exerting its action on gelatin and casein when the serum is treated with chloroform. Under ordinary conditions, they believe, anti-bodies which may be destroyed by chloroform inhibit the action of the ferment. Hedin [12] has more recently found in the blood serum of the ox a weak proteolytic enzyme which acts in an alkaline medium. The enzyme is precipitated with the globulin of the serum; anti-bodies which inhibit its action are, he finds, contained mainly in the albumin fraction of the serum.

Destruction of the anti-enzymotic action of the blood serum by acetic acid is in accord with observations of Levene,[13] who has shown that the resistance which uncoagulated egg-albumin and normal blood serum offer to digestion by an extract of fresh pancreatic tissue is overcome by exposing these substances to the action of acetic acid before addition of the extract.

Anti-enzymotic Action of the Serum of the Blood.—The well-known fact that certain anti-ferments are present in the normal blood serum has suggested the probability that the anti-body which in the serum of a sterile inflammatory exudate inhibits the activity of ferment in the leucocytes is derived from the serum of the blood. The action of blood serum of the dog was therefore compared with the serum of an exudate obtained by injecting aleuronat into the pleural cavity.

Experiment 17.—An exudate was obtained by injecting aleuronat into the pleural cavity of a dog, and the cells removed by centrifugalization were suspended in nineteen times their volume of salt solution. In order to increase the amount of digestible proteid, into each flask were put 5 c. c. of diluted blood serum coagulated by heating to 100° C. The carefully sealed flasks containing toluol were kept at 37° C. for five days. Blood serum was obtained by centrifugalization of the defibrinated blood.

(Cells + blood serum) coagulated as control.....	3.45 c. c.
Cells + coagulated blood serum at 37° C. 5 days.....	10.95 "

[11] *Compt. rend. de la Soc. de Biol.*, 1903, lv, 327, 690, 693.

[12] *Jour. of Phys.*, 1904, xxx, 195.

[13] *Jour. of Med. Research*, 1903, x, 217.

Cells + coagulated blood serum + 5 c. c. exuded serum at 37° C. 5 days	8.5 c. c.
Cells + coagulated blood serum + 5 c. c. blood serum at 37° C. 5 days	8.25 "

When it is recalled that the serum of the exudate contains a considerable amount of nitrogen in a form not coagulable by heat, it is evident that inhibition of digestion is more marked than that indicated by these figures. The inhibitory action of blood serum and of serum from the inflammatory exudate is, it appears, approximately equal. In order to compare more accurately the effect of serum from the blood and from the exudate, another series of mixtures was prepared from the blood serum and exuded serum of an animal which had received an intrapleural injection of aleuronat.

Experiment 18.—Washed cells from an exudate obtained two days after the injection of aleuronat were suspended in nine times their volume of salt solution. Each flask contained 5 c. c. suspension of cells and 5 c. c. exuded serum or 5 c. c. blood serum, and was kept at 37° C. for five days.

(Cells + exuded serum) coagulated as control	6.4 c. c.
Cells + exuded serum at 37° C. 5 days	7.9 "
Cells + coagulated exuded serum at 37° C. 5 days	21.55 "
(Cells + blood serum) coagulated as control	6.25 c. c.
Cells + blood serum at 37° C. 5 days	8.35 "
Cells + coagulated blood serum at 37° C. 5 days	31.6 "

The experiment demonstrates that the anti-enzymotic action exhibited by the serum of the exudate is exerted by the serum of the blood as well, and in the case of a sterile exudate two days after the onset of inflammation in approximately the same degree.

Anti-enzymotic Action of the Serum of Bacterial Exudates.—An accidental infection of the pleural cavity of the dog afforded an opportunity for the study of an exudate containing bacteria. Following aspiration of the right pleural cavity twenty-four hours after the injection of aleuronat, breathing became laborious and the animal appeared sick. By aspiration three days later, about 150 cubic centimeters of opaque grayish-white fluid were withdrawn from the right pleural cavity. Cultures from the

exudate showed the presence of streptococcus pyogenes in immense numbers, accompanied by a few colonies of a coccus which proved to have the cultural characters of staphylococcus pyogenes albus. Cover-slip preparations from the exudate contained cocci in great numbers, situated largely outside of the leucocytes. The animal died shortly after the removal, by aspiration, of 150 cubic centimeters of exudate.

EXPERIMENT 19.—Cells from the exudate containing streptococcus pyogenes and staphylococcus pyogenes albus were separated from the serum by centrifugalization and suspended in approximately nine times their volume of salt solution. Mixtures of cells and serum were made, and to each were added 5 c. c. toluol. Cultures made from each flask at the end of incubation remained sterile.

5 c. c. cells at 37° C. 5 days	6.05 c. c.
Control	2.85 "
Digestion	3.20 "
5 c. c. serum at 37° C. 5 days	8.25 "
Control (coagulated immediately)	7.7 "
5 c. c. cells + 1 c. c. serum at 37° C. 5 days	7.25 "
Uncoagulable nitrogen in 5 c. c. cells (2.85 c. c.) and 1 c. c. serum (1.54 c. c.) represented by	4.39 "
Digestion	2.86 "
5 c. c. cells + 1 c. c. coagulated serum at 37° C. 5 days	8.65 "
Uncoagulable nitrogen in 5 c. c. cells and 1 c. c. serum represented by	4.39 "
Digestion	4.26 "
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	12.15 "
Uncoagulable nitrogen in 5 c. c. cells (2.85 c. c.) and 5 c. c. serum (7.7 c. c.) represented by	10.55 "
Digestion	1.60 "
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	21.85 "
Uncoagulable nitrogen in 5 c. c. cells and 5 c. c. serum represented by	10.55 "
Digestion	11.30 "

The anti-enzymotic action exhibited by the serum of a sterile inflammatory exudate has been demonstrable in an exudate containing bacteria in considerable number. Inhibition of en-

zymotic action is, however, less complete than in many of the experiments previously recorded. This difference may be partially referable to the fact that the leucocytes in this experiment were less thoroughly washed with salt solution than were those used in other experiments.

In the following experiment an exudate obtained by injecting bacillus coli into the pleural cavity of a dog was used. The serum of the exudate exerted an inhibitory effect upon the cellular ferments, but in the amount previously used the serum failed to prevent completely digestion.

EXPERIMENT 20.—Two entire cultures of bacillus coli grown forty-eight hours on agar-agar surfaces 7 × 14 cm. were injected into the right pleural cavity of a dog. Twenty-two hours later 30 c. c. of turbid reddish fluid were withdrawn by aspiration. Carefully washed cells in nine times their volume of salt solution were subjected to autolysis under toluol in the following mixtures:

5 c. c. cells at 37° C. 5 days	7.1	c. c.
Control	2.25	"
Digestion	4.85	
5 c. c. cells + 5 c. c. serum at 37° C. 5 days	7.35	"
(5 c. c. cells + 5 c. c. serum) coagulated immediately as control	4.55	"
Digestion	2.80	"
5 c. c. cells + 5 c. c. coagulated serum at 37° C. 5 days	18.7	"
(5 c. c. cells + 5 c. c. serum) coagulated immediately as control	4.55	"
Digestion	14.15	"

Cultures made from each mixture at the end of incubation remained sterile, although the exudate on removal from the pleural cavity contained bacillus coli, recovered by culture.

Changes Produced by Incubation in the Serum of Inflammatory Exudates.—A fact repeatedly observed during the course of these experiments requires some notice. When exudates removed one or two days after the onset of inflammation are subjected to autolysis, less nitrogen is demonstrable in the uncoagulable filtrate after digestion for from two to five days than in the control prepared by coagulating the fresh exudate. This fact is illustrated by Experiments 1, 2, 3, 4, 6, 7, in which this difference is at times considerable, representing in Experiments 1, 2, 3, and

6 (aseptic digestion) from one-fifth to one-eighth of the nitrogen present in the filtrate from the fresh exudate. Subsequent experiments (Nos. 8, 10, 11, and 14) show that this difference is referable to the serum alone.

	Uncoagulable nitrogen in fresh serum of exudate represented by	Uncoagulable nitrogen in serum of exudate after incubation at 37° C. represented by
Experiment 8....	4.15 c. c.	3.7 c. c.
" 10....	4.5 "	3.85 "
" 11....	7.25 "	6.5 "
" 14....	6.3 "	4.6 "

In Experiment 10, a mixture of cells and serum confirmed the result obtained with serum alone. An exception to the facts just described is afforded by Experiment 14, in which the exudate was removed from the pleural cavity three days after the onset of inflammation, and by Experiment 19, in which the serum of the exudate contained streptococci in great numbers. The explanation of this disappearance of uncoagulable nitrogen is not evident. There is little probability that nitrogen has escaped as ammonia formed by digestion. It is not inconceivable that proteids have been less completely coagulated in the control than in the specimen incubated at 37° C., but there is no direct evidence of such a difference. Transformation of soluble nitrogen-containing substances into a form precipitable by heat is obviously not demonstrable by the methods employed.

Anti-enzymes.—Anti-bodies to several enzymes have been found in the normal blood serum. Hammarsten and Röden [14] demonstrated that the blood serum of the horse inhibits the action of lab-ferment and thus prevents coagulation of milk. The phenomenon has been studied by Briot,[15] Morgenroth,[16] and Fuld and Spiro.[17] The anti-tryptic action of the blood serum was first noted by M. Hahn [18] and later studied by a considerable

[14] *Upsala Läkareforenings forhandlingar*, xxii, 546; Ref. Maly's *Jahresb.*, 1887, xvii, 160.

[15] *Compt. rend. de l'Acad. des Sciences*, 1899, cxxviii, 1359.

[16] *Cent. für Bakt.*, 1899, xxvi, Abt. i, 349; 1900, xxvii, Abt. i, 721.

[17] *Zeit. für phys. Chem.*, 1900, xxxi, 132.

[18] *Berliner klin. Woch.*, 1897, xxxiv, 499.

number of observers, Landsteiner,[19] Glaessner,[20] and Cathart [21] having sought to determine to what fraction of the serum the anti-body is attached. Ascoli and Bezzola [22] have described an increase of the anti-tryptic action of the blood serum during the early stage of pneumonia, followed by a gradual return to normal after the crisis. Delezenne [23] maintains that the anti-tryptic action of the blood serum is referable to an anti-enterokynase preventing the amboceptor-like action of enterokynase. Bayliss and Starling,[24] believing with Pawlow that enterokynase is a ferment converting trypsinogen into trypsin, oppose the view of Delezenne and furnish evidence to prove the existence of a true anti-trypsin. In a limited number of individuals they find that the serum of the rabbit contains anti-enterokynase.

Hedin [25] has demonstrated the presence in the spleen of two ferments, one acting in an acid the other in an alkaline medium; in the serum of the ox he has found an anti-body inhibiting the action of the latter and attached mainly to the albumin fraction of the serum.

The foregoing experiments show that the ferments contained in the leucocytes are incapable of acting in the serum of the exudates employed. The activity of these ferments is apparently limited to the cells which produce them. Solid particles ingested by the cells and thus separated from the serum are acted upon by these ferments. By phagocytosis substances insoluble in the fluids of the body are brought into contact with powerful ferments capable if unchecked of destroying tissues. Destruction of tissue occurs within an abscess when with the formation of pus, as the result perhaps of loss of anti-enzymotic action, fibrin and necrotic tissue undergo solution. In the later stages of sterile inflammation and with bacterial infection of a serous cavity some loss of anti-enzymotic power has been demonstrable.

[19] *Cent. für Bakt.*, 1900, xxvii, Abt. i, 357.
[20] Hofmeister's *Beiträge zür chem. Phys.*, 1903, iv, 79.
[21] *Jour. of Phys.*, 1904, xxxi, 497.
[22] *Berliner klin. Woch.*, 1903, xl, 391.
[23] *Compt. rend. de la Soc. de Biol.*, 1903, lv, 132.
[24] *Jour. of Phys.*, 1905, xxxii, 129.
[25] *Ibid.*, 1904, xxx, 155.

It is not improbable that the number of anti-enzymes is greater than that now recognized. Facts already known suggest that such bodies accompany many ferments, holding them in check and limiting their action to those situations in which their proper function is accomplished.

CONCLUSIONS.

The serum of an inflammatory exudate has the power of inhibiting the action of proteolytic ferments contained in the leucocytes. This anti-enzymotic power is possessed by the blood serum from which it doubtless passes into the exudate.

In the later stages of inflammation there is some diminution of this anti-enzymotic action.

The anti-body contained in the serum is destroyed by a temperature of 75° C.

The proteolytic ferments of the leucocytes act both in an acid and in an alkaline medium but are most efficient in the latter. The anti-enzymotic action of the serum is favored by an alkaline reaction, but is completely lost in an acid medium.